1972

Byssochlamys Fulva: Nutrition of Ascospore Formation; Fatty-Acid Profiles of Ascospores, Conidia, and Mycelia; And Electron-Microscopy of Ascospores and Conidia.

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HEBERT, Richard Joseph, 1940-
BYSSOCHLAMYS FULVA: NUTRITION OF ASCOSPORE FORMATION; FATTY ACID PROFILES OF ASCOSPORES, CONIDIA, AND MYCELIA; AND ELECTRON MICROSCOPY OF ASCOSPORES AND CONIDIA.

The Louisiana State University and Agricultural and Mechanical College, Ph.D., 1972
Microbiology

Byssochlamys fulva: nutrition of ascospore formation; fatty acid profiles of ascospores, conidia, and mycelia; and electron microscopy of ascospores and conidia

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
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August, 1972

Acknowledgment

The author wishes to extend a sincere thank you to Dr. A. D. Larson who patiently directed this study. He also wishes to thank Dr. L. T. Hart and Dr. G. T. Dimopoullos for their guidance and encouragement when it was needed. He also wishes to thank Dr. V. R. Srinivasan and Dr. H. D. Braymer who were always willing to give advice. He also appreciates the time generously invested in him by all the members of the Department of Microbiology -- faculty, students and staff.

A very special thank you to Miss Marsha White and Mrs Gayle LoPiccolo for their help with the electron microscopy. Also a very special thank you to Carl Davis, Marvin Fleenor, Elizabeth Eubanks and C. K. Banerjee who were always willing to exchange ideas.

Most of all, the author wishes to express his gratitude to his wife Georgia Ann and his children Brian and Deborah, to whom this dissertation is dedicated.

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Abstract

Byssochlamys fulva strains derived from single ascospores showed B. fulva to be homothallic. Nutritional and asci production studies revealed that the strains consisted of four distinct phenotypes. Extreme variation in the percent of asci which could be induced to germinate by heat shock at 70 C for two hours and the amount of asci produced by each strain was evident.

A synthetic medium was devised which could be utilized in liquid form to study asci production or in solid form (2% agar added) to study variation between strains in terms of growth rate, asci production, and pigmentation.

Lipid analysis of B. fulva ascospores showed the ascospores to be 22.75% lipid on a dry weight basis. Seventy-five percent of the lipid was located in the sporoplasm. Ten percent of the ascospore coat was found to be lipid. The ascospore coat accounted for approximately 60% of the ascospore dry weight. Lipid class analysis of the ascospores revealed phospholipids, monoglycerides, diglycerides, triglycerides, free fatty acids, hydrocarbons, and two unknowns.

Fatty acid comparisons of asci, conidia and hyphae revealed the asci contained more saturated fatty acids with carbon chain lengths of 19 or longer than did the conidia and hyphae. Analysis of the fatty acids within the asco-

spores revealed that approximately 60% of the saturated fatty acids were located in the sporoplasm. The fatty acids with chain lengths greater than 19 carbons were C20. unk. #1, unk. #2, C21, unk. #3, C22, unk. #4, br-C23, C23, unk. #5, br-C24, unk. #6, unk. #7, unk. #8, unk. #9, br-C26, unk. #10, C27, unk. #11, unk. #12. Unknown #8 was the only unsaturated fatty acid in this series and was present in the greatest concentration in the sporoplasm. Of this long chain group, unk. #3, br-C24, unk. #10, C27, unk. #11, and unk. #12 predominated in the spore coat. The remaining saturated fatty acids of this series and those from C11 through C18 predominated in the sporoplasm.

Fatty acid comparisons of the individual lipid classes revealed each lipid class had a distinctive fatty acid pattern. The only two classes which had similar patterns were the monoglycerides and phospholipids. Comparisons of fatty acids present in sporoplasm and ascospore coats within each lipid class revealed that only the diglycerides exhibited significantly different fatty acid contents.

Comparisons of the ultra-structure of the conidia and ascospores showed that the ascospores had a much thicker spore coat and more condensed cytoplasm than did the conidia. The ascospores also exhibited a folded structure of cytoplasmic membrane which is not typical of ascospores

of the class Ascomycetes. The conidia possessed more structural differentiation than the ascospores.

INTRODUCTION

The genus Byssochlamys, a soil ascomycete (Put, 1964; Splittstoesser et al., 1971), was first constructed by Westling in 1909. The genus contains two species -- B. fulva and B. nivea. The former species, when grown at 30 C or above on Czapek's agar, is buff in color and characterized by the production of 8-spored asci which are conglomerate, globose to subglobose, 8.5 - 10.5 x 7.5 - 9.5 u. Ascospores are hyaline and regularly ellipsoidal with dimensions of 5.5 x 3.2 to 3.8 u. The latter species on Czapek's agar is white to off white with asci and ascospores being slightly smaller than those of B. fulva. It has been suggested that this genus forms a link between the Endomycetaceae with solitary 4-spored asci, and the Gymnoascaceae, with numerous 8-spored asci surrounded by a loose web of hyphae (Brown and Smith, 1957).

During vegetative growth, Byssochlamys will convert up to 30% of the glucose utilized to mannitol. It also produces byssochlamic acid which exhibits a toxicity for mice (Raistruck and Smith, 1933) and an unknown substance toxic for chicks (Chu, 1969). Many degradative enzymes are produced by the fungus, some of which are pectinolytic (Put and Kruiswijk, 1964). Some enzymes reported to be produced by Byssochlamys are polygalacturonase, pectinesterase, arabanase, galactanase (Reid, 1951), pectin transeliminase,

endopolygalacturonate lyase, and endopolymethygalacturonate lyase (Chu, 1969).

The ascospores of the genus are extremely interesting structures which are resistant to a number of influences lethal to most fungi. The ascospores can tolerate up to 50 parts per million of SO_4 in saline and plum syrup, and up to 450 ppm in a solution containing 10% sucrose and 1.0% peptone. The ascospores are not killed by high concentrations of ammonia or by 1:200 acetaldehyde (Brown and Smith, 1957). The ascospores in absolute alcohol remain viable for at least 30 weeks (Raistruck and Smith, 1933; Olliver and Rendle, 1934).

Another important characteristic of Byssochlamys ascospores is their dormancy. All attempts to induce germination have failed to consistently approach 100% germination. It has been shown that heat shock alone (Hull, 1939: Splittstoesser et al., 1969) or heat shock in combination with inducers do not consistently give good germination values (Yates et al., 1968). Heat shock induced germination of ascospores of either species does not exceed 30% of the population. The highest germination values for ascospores of B. nivea are obtained at pH 3.5 to 4.0 with a heat shock of 75 C for five minutes. Soluble starch or ascorbate significantly supresses germination of B. nivea ascospores. A combination of heat shock, aeration and acetate will give 82% to 84% germination. However, there

is considerable fluctuation with different preparations. The variation has not been alleviated by standardization of harvest procedures, the age of the culture at harvest, varying the humidity during growth and ascospore production or by the use of various growth media. Furfural and ethanol, which induce germination of Neurospora ascospores, have no effect on B. nivea ascospores (Yates et al., 1968).

An important characteristic of the Byssochlamys ascospores is their ability to withstand thermal inactivation for extended periods of time. Thermal death-point values of 93.3 C (Williams et al., 1941) and 96.6 C (Hull, 1939) have been reported. Because of this thermal resistance, the genus Byssochlamys has become important to the food industry where contamination of processed fruits has resulted in considerable loss of products (Maunder, 1969). Destruction of fruit by the organism is putrefactive, resulting in loss of consumer value (Put and Kruiswijk, 1964).

The first incidence of food spoilage caused by Byssochlamys was reported by Olliver and Smith in 1933. The incidence of Byssochlamys species in food spoilage seems to be increasing. Originally considered to be present primarily in Great Britain (Olliver and Rendle, 1934; Raper and Thom, 1949), it has recently been demonstrated in Switzerland (Lüthi et al., 1961), Canada (Yates and Ferguson, 1963), the United States (Michener et al., 1966), Australia

(Spurgin, 1964), and Denmark (Jensen, 1960).

The formation of heat resistant ascospores can take place under aerobic conditions (Brown and Smith, 1957) or anaerobic conditions (Yates et al., 1963). The optimal pH for ascospore production on non-defined media is 2.0 to 3.0 (Splittstoesser et al., 1969). A thorough study of heat resistance and heat inactivation of ascospores has been hindered by the presence of notable diverse heat resistant populations as shown by Hull in 1939 and confirmed by Thompson in 1969. This significant phenomenon is characterized by a second heat activation after the originally heat shocked population has been killed. Thompson (1969) showed that the second population is activated after 25 minutes at 92 C.

An important step in studying heat resistance of B. fulva ascospores has been the attempt by Denny and Brown (1969) to correlate heat resistance with pigmentation of strains. They showed a five-fold increase in heat resistance of buff strains over white strains. However, they did not consider the possibility that their white strains could be B. nivea, even though the differential classification of the two species is based primarily on buff and white pigmentation (Brown and Smith, 1957). It is generally accepted that B. nivea is less heat resistant than B. fulva (Yates, 1963).

For control of Byssochlamys in the food industry

several techniques have been suggested. These include heating at 90 C (Hull, 1939), filtration (King et al., 1969) and the use of SO_2 (Gillespy, 1940; Gillespy, 1946). A high temperature for a short period of time has been the best solution to the problem at the present time.

Essentially pure preparations of ascospores and asci are necessary for studying heat resistance. Filtration (Partsch, 1969) and differential centrifugation are currently employed to obtain purified asci or ascospore preparations. However, neither of these methods alone gives desired or consistent results (Splittstoesser, personal communication).

For detection and enumeration of Byssochlamys sp., heat shocking at 75 C for 1 hour (Gillespy, 1936; Gillespy, 1938; Splittstoesser, 1971) or at 85 C for 10 minutes (Yates, 1969) followed by plating on complex media (Canada, 1969) is the procedure most commonly used. No synthetic plating medium has been developed although Czapek's agar is generally used for classification (Brown and Smith, 1957).

No genetic analysis to determine whether Byssochlamys sp. are homothallic or heterothallic has been reported. Neither ultrastructural comparisons of conidia and ascospores nor studies of ascosporulation in a liquid synthetic medium have been reported.

Recently, there has been an interest in the fatty acid

composition of thermophilic fungi in relation to heat resistance (Mumma et al., 1970; Mumma et al., 1971; Mumma et al., 1971a). No studies on the fatty acid composition of B. fulva have been reported.

The studies recorded in this dissertation were primarily concerned with the following aspects of B. fulva:

1. The isolation of single ascospores to determine if B. fulva is heterothallic or homothallic.
2. The fabrication of a liquid synthetic medium suitable for ascospore production.
3. Studies of ascospore production by a strain which produces large numbers of asci in the liquid synthetic medium.
4. An attempt to correlate spore production with pigmentation of the forty strains isolated from the parent culture.
5. The study of ascospore dormancy and maturation in asci for the population heat activated by 70 C for two hours.
6. The refinement of a purification procedure to obtain essentially pure asci preparations.
7. The development of a technique for obtaining essentially pure ascospore preparations from purified asci.
8. The following aspects of lipid composition:
   a. Comparison of the fatty acid profiles of asci, conidia and hyphae.
   b. Comparative fatty acid profiles of ascospore coats

and sporoplasm.

c. Determination of the fatty acid profiles of lipid classes derived from ascospore coats and sporoplasm.

MATERIALS AND METHODS

I. Organism

Byssochlamys fulva (National Canners Association strain 68-10) was obtained from Dr. Paul J. Thompson (Gerber Baby Foods, Freemont Michigan). Stock cultures were maintained on potato dextrose agar (Difco) (PDA) slants at room temperature and transferred annually.

II. Preparation of single ascospore suspensions

1. An aqueous suspension of a known concentration of asci was sedimented in a 25 ml screw cap centrifuge tube so that a firm pellet was obtained.
2. The supernatant fluid was carefully removed by aspiration.
3. Approximately 4.0 grams of glass beads (0.1 - 0.11 mm) were then added to the tube.
4. The mixture was agitated on a Vortex mixer for 1.5 to 2.0 minutes at maximum speed in order to rupture the asci.
5. Water was then added until the original volume was obtained and the contents of the tube were suspended by inverting the tube several times.
6. The glass beads were allowed to settle and counts were made of asci and ascospores with a hemocy-

tometer to determine percent recovery of asci and ascospores.

III. Isolation and characterization of Byssochlamys fulva strains

A. Strain isolations for nutritional studies

Purified ascospore suspensions were spread on microscope glass slides overlaid with 3.0% agar and individual ascospores were picked with fine glass needles with the aid of a microscope at a magnification of 100X. One hundred thirty two ascospores were picked. Each ascospore was placed in 1 ml of potato dextrose broth (PDB) in separate screw cap tubes.

The ascospores were heat shocked at 85 C for 5 minutes by placing the tubes in a water bath. The tubes were then cooled in ice water and incubated at 34 C. Care was taken to insure the caps were securely tightened.

After four days of incubation the tubes exhibiting growth were removed and the tubes without growth were retreated as above. This process was repeated five times. Each time the tubes with growth were removed and the tubes without growth retreated. After the fifth treatment, the remaining tubes without growth were heat shocked for 15 minutes at 85 C and incubated as before. After this final treatment all tubes which showed no growth were discarded.

Strains were coded to describe the accumulative time

of heat shock necessary to induce germination; for example, Bf-1-5 indicates Byssochlamys fulva strain 1 isolated after 5 minutes heat shock at 85 C under the conditions described.

B. Growth studies and asci production of strains isolated.

The isolates were preserved by lyophilization of conidia. For preparation of inocula, lyophilized conidia were seeded onto potato dextrose agar (PDA) in petri plates and the plates were incubated until conidia were formed. Conidia were always used for inoculation of media. Plates were inoculated by placing a loopful of a water suspension of conidia at the center of each plate.

The first growth study was carried out on PDA. Plates were inoculated in triplicate and scored for growth rate, pigmentation and asci production. An attempt was made to group the strains according to the method of Denny and Brown (1969), which is based on pigmentation.

The second growth study was carried out on a modified Czapek's agar consisting of Czapek's basal salts mixture plus sucrose (20.0 g/l), calcium chloride (0.55 g/l), riboflavin (9.0 mg/l), nicotinamide (100 mg/l), ascorbate (200 mg/l) and agar (20.0 g/l). The medium was adjusted to pH 4.0 before autoclaving.

C. Heat shock activation percentages of some strains.

Eight of the isolated strains were examined for the percent germination of asci after heat shock at 70 C for 2

hours. Asci were obtained from cultures grown on PDA. The asci concentration was the same in all experiments. Strains 5, 6, 7, 10, 12, 14, 19, and 25 were examined.

D. Heat shock experiments with single ascospore suspensions.

Strains Bf-25-10 and Bf-19-10 were used in this study because they were typical of the two species in Byssochlamys as described by Brown and Smith (1957). Asci from 14, 19, and 40 day old cultures of Bf-25-10 and asci from a 40 day old culture of Bf-19-10 were ruptured as described above and the liberated ascospores were heated at 70 C in 0.01 M phosphate buffer (pH 7.0) for two hours and then plated on PDA to determine the percent germination.

IV. Synthetic liquid medium for asci production

Strain Bf-25-10 was employed to measure asci production on the liquid synthetic medium described below. Conidia were used for inoculation of media. Inocula was prepared by subculturing three times at three day intervals on potato extract agar with 2.0% sucrose (PSA) or 2.0% dextrose (PDA) and the conidia from the plates of the third transfer were collected. Conidia were harvested by first flooding the surface of the plates with sterile distilled water followed by scraping the surface of the culture with a sterile bent glass rod. The material was then filtered through sterile cheesecloth to remove mycelia and the coni-

dia were washed three times by centrifugation in sterile distilled water. Inocula of conidia was used the day of preparation. A hemocytometer was employed to perform microscopic counts of conidia and no attempt was made to differentiate between microconidia and macroconidia.

Czapek's salts mixture with 5.5 g $CaCl_2$, 9.0 mg riboflavin, 100 mg nicotinamide, 200 mg ascorbic acid added per liter was the basal synthetic medium. Glucose (2.0%) or sucrose (2.0%) was provided as the major carbon source for synthetic and potato extract media. Synthetic medium with glucose and the other media employed were adjusted to an initial pH of 4.0. The synthetic media were inoculated with conidia in order to give a concentration of 50 to 500 per ml of medium. Only inocula giving 50 conidia per ml of final concentration were employed for media containing potato extract. Media was dispensed in 50 ml volumes in 250 ml flasks with cotton plugs. All pH measurements were done with a Corning model 12 pH meter. A precipitate was noted upon cooling after autoclaving of the synthetic medium, but the precipitate dissolved as fungal growth ensued.

Cultures were incubated at 34 C and mycelial mats from duplicate flasks were harvested at selected intervals for determination of dry weights and asci counts. In each experiment the pH of the residual medium was determined. For these determinations mats were removed with a bent glass rod and homogenized in 20 ml of distilled water in a Sorvall

omnimixer operated at maximum speed for 7 minutes with the container immersed in an ice water bath. The final volume was then noted and asci counts were done with a hemocytometer. Ten ml were removed and evaporated to dryness at 110 C in tared aluminum weighing pans for dry weight determinations. The particulate material in the remaining homogenate was then washed three times in distilled water and employed for heat activation studies. No attempt was made to rupture the asci, therefore heat activation results were obtained with intact asci. Suitable dilutions of asci were plated on PDA to include the range of 1.0% to 100% activation based on microscopic counts. For heat activation, asci were heated in 0.01 M phosphate buffer (pH 7.0) for two hours and plated on PDA to determine the percent germination. Asci counts were performed with a hemocytometer before and after heating to determine if there was any significant breakage of asci.

V. Preparation of purified asci suspensions

The growth (14 to 19 days incubation) from 300 PDA plates was harvested with distilled water. The material was homogenized in an omnimixer as previously described. The particulate material was washed by centrifugation three times in distilled water. The resulting pellets were suspended in two volumes of distilled water and placed in a refrigerator for two to three months to allow autolysis of

hyphae to occur.

Prior to purification, the particulate material was washed three times in distilled water. The supernatant material was discarded and the pellets containing asci, conidia and hyphal fragments were retained. Asci at a concentration of 10^6 per ml were suspended in 300 ml of distilled water containing 0.1% Tween 80 and carefully layered over an equal volume of 50% sucrose containing 0.1% Tween 80. After settling by gravity for 4 to 6 hours, the contents of the bottle were removed from the top by aspiration into 3 equal portions. The upper layer was discarded and the bottom layer was stored at 4 C. The asci and conidia in the middle layer were counted and sedimented by centrifugation (6,000 - 12,000 x g). The supernatant fluid was discarded, the pellet was resuspended in 300 ml of distilled water containing 0.1% Tween 80 and again layered over 50% sucrose containing 0.1% Tween 80. The entire process was repeated twice more or until virtually all of the asci in the original suspension could be accounted for in the bottom layers. All of the bottom layers were then pooled. This suspension was then diluted with an equal volume of distilled water and dispensed into 50 ml centrifuge tubes. Particulate material was sedimented at 500 rpm in an International refrigerated centrifuge, using a swinging bucket head, for five minutes. Half the supernatant fluid was removed from each tube, distilled water added to attain the

original volume, and particulate matter was resuspended. The particulate material was again sedimented at 500 rpm employing a swinging bucket head. This washing process was repeated 6 to 10 times. Finally, the particulate material was washed in distilled water 5 more times in order to insure removal of the last traces of sucrose and Tween 80.

The washed pellets from the low speed centrifugation prodedure were suspended in distilled water and transferred to 50 ml polyporpylene centrifuge tubes. The suspensions were sedimented at 6,000 x g to 12,000 x g for five minutes using the Sorvall RC-2B centrifuge and the SS-34 head. The loosely packed material on the surface of the pellets of asci was removed by gently agitation with distilled water. The material was retreated at high speed to recover as many asci as possible.

The pellets of purified asci thus obtained were saved for electron microscopy and for lipid analysis.

Photomicrographs were made of the original material and the final asci preparation.

VI. Lipid analysis: fatty acid profiles of asci, conidia and hyphae

Purified asci suspensions were obtained as described in part IV of the Materials and Methods.

Purified conidia were obtained from culture on PDA plates incubated for 3 days at 34 C as follows. Growth was

scraped from the surface of the culture into distilled water with a bent glass rod and was then filtered through cheesecloth and the conidia were washed three times with distilled water by centrifugation.

Hyphae were obtained by inoculating 500 ml of PDB contained in a 1 liter flask with conidia and incubating the culture on a rotary shaker at 34 C for 3 days to minimize conidiation. The hyphae were harvested by filtering the culture through cheesecloth. The hyphae retained by the cheesecloth were washed with sterile distilled water to remove contaminated conidia and medium as completely as possible.

The lipids of asci, conidia, and hyphae were extracted with chloroform:methanol (2/1 v/v) for four days at 25 C with stirring (Folch et al., 1957). The residue was removed by filtration through ether-extracted Whatman #1 filter paper. The filtrate was washed with water in a separatory funnel. The non-aqueous portions were pooled and dried over anhydrous Na_2SO_4 under N_2 at 4 C.

The residue was re-extracted with chloroform:methanol (2/1 v/v) by refluxing for two days and the lipid was processes as before.

The chloroform was evaporated under N_2 and the extracted lipid was transmethylated according to the method of (Stoffel et al., 1959) with the modification that H_2SO_4 was used instead of HCl. Ten ml of petroleum ether and water

were added and after mixing the aqueous layer was removed. This washing was repeated four times in order to insure complete removal of the sulfuric acid. The petroleum ether fraction was dried over anhydrous Na_2SO_4 for subsequent analysis by gas chromatography.

The F. & M. Gas Chromatograph, Model 700 equipped with a dual flame ionization detector and dual columns was employed. The column packing was 8.0% ethylene glycol adipate on Chromport A, 80 - 90 mesh in 10 foot stainless steel columns maintained at 185 C. The injection port temperature was 300 C, the detector temperature was 280 C, and the carrier gas flow (helium) was 60 ml/min.

Fatty acid esters were identified by comparison of retention times with methyl ester standards (Hormel Institute) and by comparing with logarithmic plots of retention ratios (Preston and Spreckelmeyer, 1971). Bromination (Preston and Spreckelmeyer, 1971) was used to obtain further evidence of the unsaturated fatty acid esters. Trimethyl silyation (Sweely et al., 1963) of the preparation was employed to determine the absence or presence of alcohols. Triangulation was employed for determining the areas under the peaks (Preston and Spreckelmeyer, 1971).

VII. Analysis of the ascospores

A. Gravimetric determination of percent spore coat per ascospore

Asci (45 mg) were harvested as described in part V of the Materials and Methods and asci and ascospores were ruptured with a Nossal cell disintegrator (Horikoshi and Iida, 1964). The glass beads were allowed to settle and the remaining material sedimented at 5,000 rpm for 20 minutes in the Sorvall RC-2B with a small angle head. In this manner a soluble fraction presumably consisting mainly of spore cytoplasmic (sporoplasm) constituents and a non-soluble fraction, presumably consisting mainly of ascospore coats and ascus membranes, were obtained. The coats were dried at 110 C on tared millipore filter pads and weighed.

B. Quantitation of total lipids in sporoplasm and spore coats

Lipid was extracted by refluxing the supernatant material and the ascospore coats, separately, in chloroform: methanol (2/1 v/v) for two days. The extracted material was treated as previously described (part VI of Materials and Methods), and each brought to a volume of 25 ml with petroleum ether. Four separate 20 µl samples of each preparation were removed and placed in drying pans. The samples were dried under vacumn at 40 C and weighed on a Cahn electrobalance. The values obtained were used to quantitate the lipid present in each fraction.

C. Determination of lipid classes

Thin layer chromatography on silica gel G was used to

determine the lipid classes present in the sporoplasm and ascospore coats. The solvent system contained petroleum ether, diethyl ether, and formic acid in the ratio 34:45:1. Approximately equal weights of lipid were used for analysis of ascospore coat and sporoplasm lipids. After development, the plates were removed and allowed to air dry. The dried plates were sprayed with 2,7-dichlorofluorescein and lipid bands were visualized under ultraviolet light. A comparison with standards on the same plate was used for identifying the individual lipid classes.

D. Quantitation of fatty acids in sporoplasm and ascospore coat fractions

Equal amounts of lipid obtained in VII-B were removed and treated according to the procedure in part VI of the Materials and Methods. Each lipid sample was then concentrated to 0.1 ml and 9.0 µl were injected into the gas chromotograph.

A known quantity of palmitic acid standard was transmethylated and used as a standard for calculating the amount of each fatty acid species present in the unknown (Preston and Spreckelmeyer, 1971). A value of 0.478 ug of fatty acid/sq cm was obtained. All areas were quantitated at an attenuation of 200.

VIII. Fatty acid profiles of the lipid classes from the sporoplasm and ascospore coat fractions

Lipid classes were separated as described in part VII-D of the Materials and Methods and transmethylated according to part VI and the fatty acids identified by gas-liquid chromatography.

IX. Electron microscopy of ascospores and conidia

Asci and conidia were obtained as described in parts V and VI of the Materials and Methods. The following procedures were used for fixing the ascospores and conidia:

1. Ascospores and conidia were fixed in 2.0% $KMnO_4$ for 10 minutes, washed in distilled water, and post-fixed in OsO_4 for 3 to 5 hours. They were then dehydrated through a graded series of ethanol solutions. They were then transferred to a 1:1 Spur:ethanol mixture and a 3:1 mixture for 30 minutes each followed by a treatment with undiluted Spur for 1 hour and finally transferred to fresh Spur medium for 10 to 12 hours. Each mixture of spores and embedding medium was then transferred to Beem capsules and sedimented in order to obtain a compact pellet. Polymerization was carried out at 70 C for a minimum of 8 hours under vacumn.
2. The second treatment differed in that glutaraldehyde fixation was employed in place of permanganate for 10 to 12 hours.

Sections were cut on a LKB Ultratome equipped with a diamond knife, mounted upon acid treated copper grids and examined with an RCA-EMU 3 G electron microscope at 50,000 volts.

RESULTS

I. Preparation of single ascospore suspensions

It may be noted from the results presented in Tables 1a and 1 that the procedure gives good breakage of asci to yield individual ascospores with a minimal loss of ascospores. Essentially all of the ascospor·s were recovered and the theoretical colony forming units due to intact asci are insignificant.

II. Isolation and characterization of Byssochlamys fulva strains

A. Strain isolations for nutritional studies

Of the 132 ascospores isolated, 40 were induced to germinate. The germination was asynchronous in terms of the time of heat shock necessary and the number of ascospores germinating with each heat shock treatment (Table 2).

B. Growth studies of strains isolated

The forty strains isolated on PDA could be divided into four colony types with the following characteristics:

I. Initially the fungus is predominantly buff colored with the late white secondary growth usually in a granular form.

II. Growth is a mixture of buff and white. The growth may occur in the form of concentric

Table 1

Quantitative determinations of asci and ascospores in ruptured asci preparations

Sample Number	Pre-treatment counts		Post-treatment counts	
	Asci	Ascospores	Asci	Ascospores
1	1.8×10^5	1.5×10^4	2.5×10^3	1.2×10^6
2	9.2×10^4	1.0 s 10^4	2.5×10^3	5.2×10^5
3	2.5×10^5	1.2×10^5	2.5×10^3	1.3×10^6
4	1.6×10^5	1.0×10^4	6.0×10^4	6.6×10^5
5	2.7×10^5	6.2×10^4	2.5×10^3	1.9×10^6

Table 1a

Recovery of asci and ascospores from ruptured asci

Sample number	Theoretical ascospore counts	%Recovery of ascospores	% Recovery of asci	$\frac{\text{Ascospores} \times 100}{\text{asci}}$
1	1.4×10^6	85	1.3	99
2	7.2×10^5	72	2.7	99
3	2.0×10^6	65	1.0	99
4	8.0×10^5	82	37.5	91
5	1.6×10^6	118	1.0	99

Table 2

Number of strains isolated and the time of heat shock at 85 C necessary to induce germination.

Accumulative time of heat shock in minutes	Number of asco-spores germinated
5	4
10	29
15	1
20	5
25	0
40	1

rings.

III. Growth is always predominantly slightly raised and white, and it later forms a thick white mat.

IV. Growth is all white and raised. The entire inner space of the petri dish may be filled with a white cottony growth which becomes attached to the petri dish cove.

As shown in the results recorded in experiments I and II of Table 3, these growth patterns do not show good reporducibility. On PDA, asci production was relatively constant within each strain but there was variation from strain to strain (Table 4).

The following patterns designated 1, 1a, 2, and 2a were obtained on the solid synthetic medium described in part III-B of the Materials and Methods:

1. Growth is initially all white and remains white.

1a. Similar to 1 except a reddish pigment becomes visible after 4 to 8 days.

2. Growth is initially all buff with white granular growth occurring with increased incubation time.

2a. Similar to 2 except a reddish-brown pigmentation developed after 8 to 10 days.

The white growth in type 2 and 2a was initiated in the area of inoculation and eventually covered the primary growth. The secondary growth appeared separate from the

Table 3

Growth characteristics of different isolates on potato dextrose agar and synthetic medium

Strain number	Potato dextrose agar		Synthetic medium	
	Exp. I	Exp. II	Exp. III	Exp. IV
Bf-1-5	III**	III	1***	1
Bf-2-5	IV**	III	2a***	2a
Bf-3-5	II	II	1	1
Bf-4-5	III	II	1	1a***
Bf-5-5	III	III	1	1a
Bf-6-10	II**	III	1	1
Bf-7-10	I**	I	2	2a
Bf-8-10	I	I	2***	2
Bf-9-10	I	IV	1	1
Bf-10-10	II	II	1	1
Bf-11-10	I	III	1	1a
Bf-12-10	III	III	1	1
Bf-13-10	I	I	2a	2a
Bf-14-10	III	I	1	1a
Bf-15-10	I	I	2	2
Bf-16-10	I	I	2a	2a
Bf-17-10	II	II	1a	1a
Bf-18-10	I	II	2	2

Table 3 (continued)

Growth characteristics of different isolates on potato dextrose agar and synthetic medium

Strain number	Potato dextrose agar		Synthetic medium	
	Exp. I	Exp. II	Exp. III	Exp. IV
Bf-19-10	II	I	1	1
Bf-20-10	II	II	1	1
Bf-21-10	II	I	1	1
Bf-22-10	III	III	1a	1a
Bf-23-10	II	II	2	2
Bf-24-10	I	II	2	2
Bf-25-10	I	I	2	2
Bf-26-10	III	II	1	1a
Bf-27-10	II	II	1	1
Bf-28-10	III	II	2	2
Bf-29-10	I	II	2a	2a
Bf-30-10	I	I	2a	2a
Bf-31-10	II	II	1	1a
Bf-32-10	II	II	1	1
Bf-33-10	I	II	2a	2a
Bf-34-10	I	I	2a	2a
Bf-35-10	II	II	2a	2a
Bf-36-20	lost	lost	lost	lost

Table 3 (continued)

Growth characteristics of different isolates on potato dextrose agar and synthetic medium

Strain number	Potato dextrose agar		Synthetic medium	
	Exp. I	Exp. II	Exp. III	Exp. IV
Bf-37-20	III	lost*	lost	lost
Bf-38-20	I	lost	lost	lost
Bf-39-20	I	I	2a	2a
Bf-40-40	II**	I	2	2***

* - Strains lost due to contamination.

** - Roman numerals represent a code fpr the growth patterns obtained on potato dextrose agar. An interpertation is presented on page 22.

*** - Arabic numbers represent a code for growth patterns obtained on synthetic medium. An interpertation is presented on page 25.

Table 4

Asci production by different isolates on potato dextrose agar and synthetic liquid medium

Strain examined	Potato dextrose agar		Synthetic liquid medium	
	Asci production per plate		Asci production per flask	
	Plate #1	Plate #2	Flask #1	Flask #2
Bf-1-5	1.2×10^8	8.3×10^7	-	-
Bf-2-5	1.8×10^5	3.7×10^5	-	-
Bf-3-5	9.7×10^7	1.0×10^8	-	-
Bf-4-5	2.0×10^6	2.5×10^6	-	-
Bf-5-5	7.5×10^5	2.5×10^6	-	-
Bf-6-10	1.2×10^8	7.5×10^7	-	-
Bf-10-10	-	-	5.0×10^7	2.5×10^7
Bf-12-10	-	-	5.0×10^7	3.3×10^7
Bf-13-10	5.9×10^5	2.2×10^6	-	-
Bf-14-10	2.6×10^7	2.5×10^7	1.8×10^7	1.1×10^7
Bf-16-10	9.6×10^5	2.9×10^6	-	-
Bf-17-10	3.7×10^7	3.3×10^7	-	-
Bf-18-10	2.0×10^6	6.6×10^6	1.0×10^8	9.0×10^7
Bf-19-10	-	-	1.4×10^7	1.0×10^7
Bf-21-10	-	-	3.8×10^7	1.2×10^8

Table 4 (continued)

Asci production by different isolated on potato dextrose agar and synthetic liquid medium

Strain examined	Potato dextrose agar		Synthetic liquid medium	
	Asci production per plate		Asci production per flask	
	Plate #1	Plate #2	Flask #1	Flask #2
Bf-25-10	4.0×10^7	8.0×10^7	1.0×10^8	7.5×10^7
Bf-30-10	-	-	5.2×10^7	2.6×10^7

* _ Strains were not examined for asci production.

initial growth and could be removed as a discrete film.

As the results in Table 3 show, the growth patterns exhibited good reproducibility when experiments III and IV are compared. The plates in experiment III were read earlier than those of experiment IV which may acount for some of the differences in the growth patterns. After 14 days or longer, the growth patterns are definitely established.

The buff strains showed a much faster growth rate than the white strains on the synthetic agar medium. The former group covered the surface of a petri plate in 3 to 5 days, while few white strains covered the plate even on extended incubation. The white strains also showed poorer mat formation on liquid synthetic media than the buff strains. However, like the buff strains, the white strains showed consistent asci production as shown in Table 4. Variation in asci production was also noted from strain to strain.

C. Heat shock induced germination of asci from different strains

The results recorded in Table 5 indicate considerable variation in the percent of asci which can be induced by heat shock into germination.

D. Heat shock induced germination of single ascospore

Based on the percent germination of asci obtained for these strains (Table 5) it is apparent that all of the asco-

Table 5

Heat shock induced germination of asci produced by different strains.

Strain number	Growth pattern	% Germination
Bf-5-5	1a	80.43
Bf-6-10	1	11.95
Bf-7-10	2a	73.90
Bf-10-10	1	6.00
Bf-12-10	1	30.00
Bf-14-10	1a	35.00
Bf-19-10	1	50.00
Bf-25-10	2	10.00

spores within a heat activatable asci population do not germinate as indicated by the results recorded in Table 6. If all of the ascospores in the heat activatable asci population had germinated, Bf-25-10 would have given 10% germination and Bf-19-10 would have given 50% germination.

III. Synthetic liquid medium suitable for asci production

The results recorded in Fig. 1 reveal that none of the synthetic media employed in the experiment supported asci formation to the same extent of that observed with PDB or PSB. Peak asci formation was observed in 14 days with PDB and PSB whereas in the synthetic media the peak was reached in 16 to 20 days. Maximum asci production in synthetic media on a dry weight basis ranged from 30% to 50% of that obtained with PSB or PDB. After maximum asci formation occurred, there was a decrease in asci counts with PDB but no decrease was noted with PSB or in synthetic media containing sucrose as the main carbon source during the time of the experiment. If glucose was employed as the main carbon source in the synthetic media, asci formation was erratic. The data in Fig. 1 also show no appreciable difference in asci production on synthetic media when the inoculum was increased by a factor of 10 or when the calcium chloride concentration was reduced to 2.75 g/l.

The results summarized in Fig. 2 show that the dry weight per flask was slightly greater in the synthetic

Table 6

Percent heat shock induced germination of single ascospores.

Medium	Strain number	Age of culture in days	Number of ascospores	Number germinated	% Germination
PDB	Bf-25-10	14	1.4×10^7	2.2×10^5	1.5
Syn	Bf-25-10	14	8.1×10^6	2.8×10^5	3.4
Syn	Bf-25-10	19	7.2×10^6	2.2×10^5	3.0
Syn	Bf-25-10	40	2.0×10^7	1.5×10^6	7.5
Syn	Bf-19-10	40	2.2×10^7	1.5×10^6	6.8

Fig. 1. Asci production in synthetic and non-synthetic media.

A - Czapek's salts mixture with riboflavin (9.0 mg/l); nicotinamide (100 mg/l); ascorbate (200 mg/l), $CaCl_2$ (5.5 g/l) and 2% sucrose. Inoculum 50 conidia/ml.

B - Same medium as in A except 2.75 g $CaCl_2$ 1/ was used. Inoculum 50 conidia/ml.

C - Same medium as in A except inoculum 500 conidia/ml.

D - Potato extract (0.5%) with 2% sucrose. Inoculum 50 conidia/ml.

E - Potato extract (0.5%) with 2% dextrose. Inoculum 50 conidia/ml.

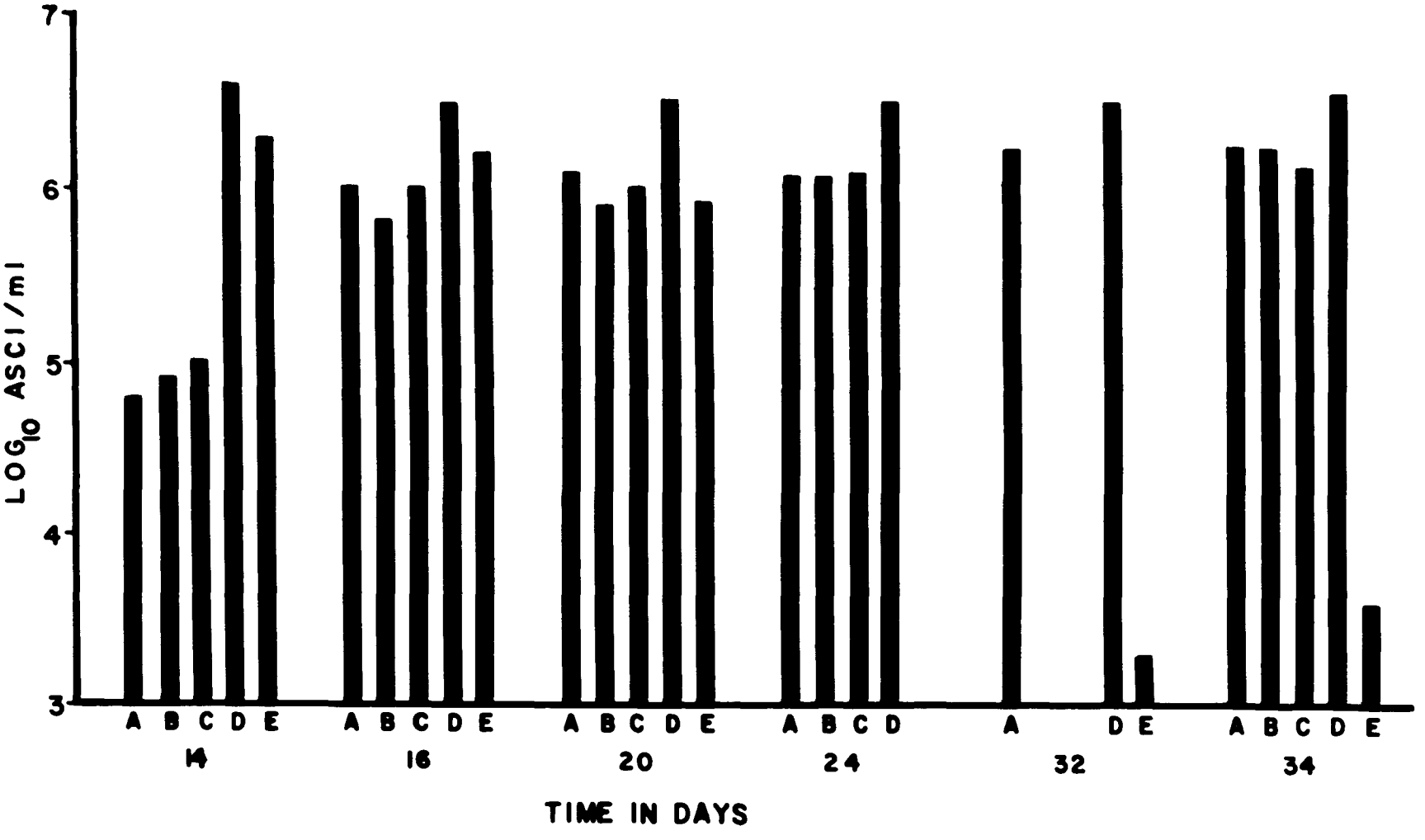
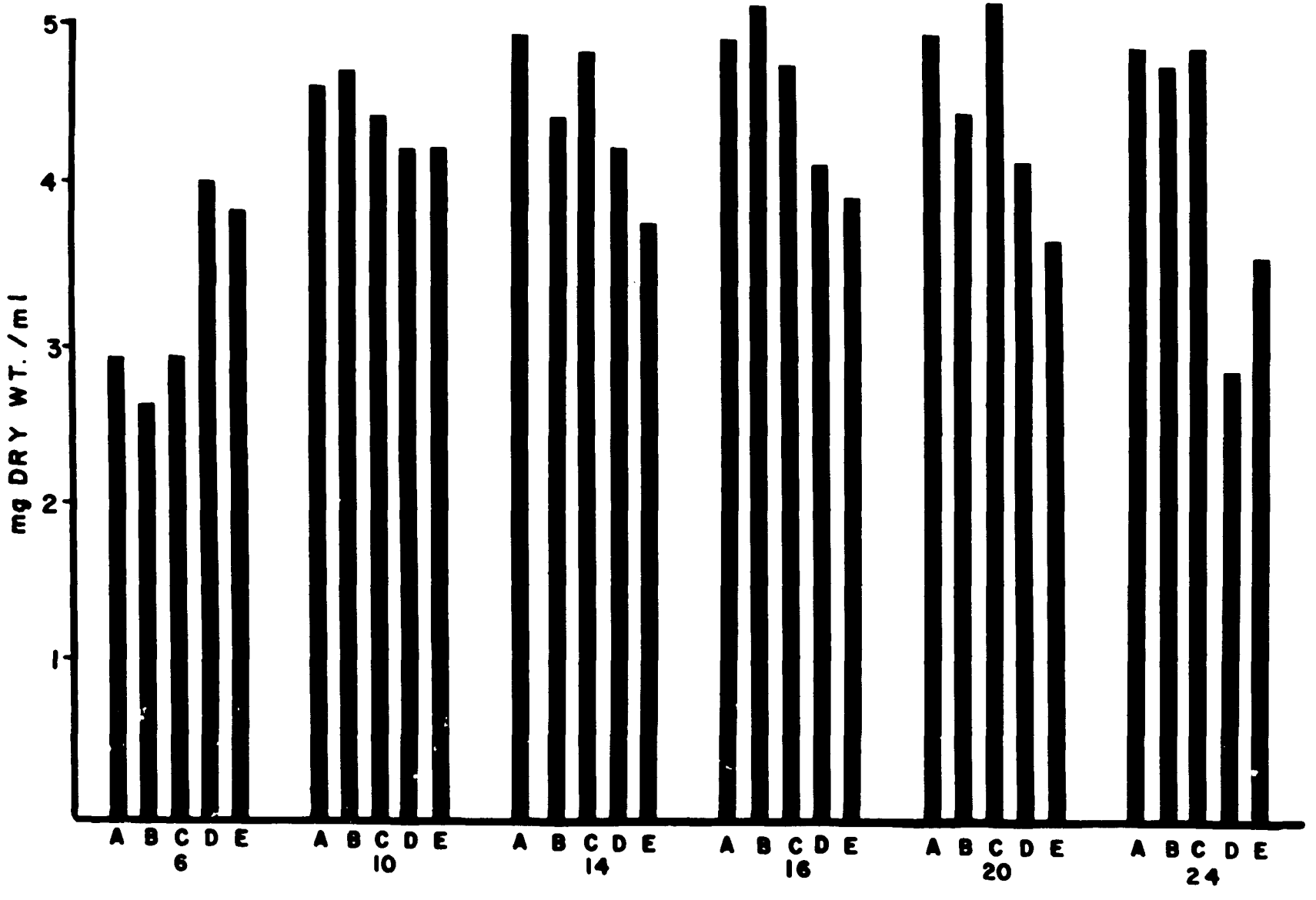

7
6
5
4
3
LOG10 ASCI/ml
A B C D E
14
A B C D E
16
A B C D E
20
A B C D
24
A
D E
32
A B C D E
34
TIME IN DAYS

Fig. 2. Dry wt/ml in synthetic and non-synthetic media. See legend of Fig. 1 for media and inocula employed.


mg DRY WT. / ml
5
4
3
2
1
A B C D E
6
A B C D E
10
A B C D E
14
A B C D E
16
A B C D E
20
A B C D E
24

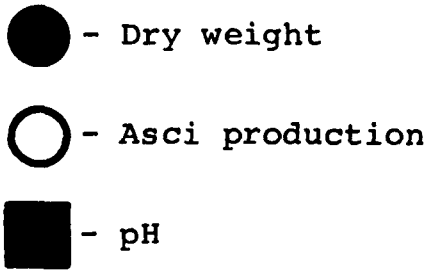
media than in the potato extract containing media. In addition, in synthetic media there was no significant difference in dry weight increase when the inoculum was increased by a factor of 10 or the calcium chloride concentration was reduced to2.75 g/l. After 20 days, autolysis of the mycelial mats became evident and therefore, dry weight determinations were not extended past 24 days. From the results shown in Fig. 1 and 2, it may be noted that the complete synthetic medium with sucrose supports the best vegetative growth and asci production.

Of the different initial pH values employed in complete synthetic medium with sucrose, only an initial pH of 4.0 gave consistent results in terms of asci production. Although the data is not reported, when an initial pH of 2.0 or 5.0 was used there was erratic asci formation. From an initial pH of 4.0, a difference in pH values occurred during growth and asci formation between PDB and PSB media. The pH with PSB stabilized after 20 days at a pH of about 5.0 while the PDB showed a steady increase in pH values between 6.0 and 7.0.

The data in Fig. 3 and 4 show that peak asci formation, dry weight maxima, and stabilization of pH occurred approximately 4 days later in synthetic medium containing sucrose as compared to PSB although the same relative patterns can be observed.

The percent of the asci population susceptible to heat

Fig. 3. Dry wt., asci production, and pH in synthetic medium composed of Czapek's salts mixture, riboflavin (9.0 mg/l), nicotinamide (100 mg/l), ascorbate (200 mg/l). $CaCl_2$ (5.5 g/l) and 2% sucrose. Inoculum 50 conidia/ml.



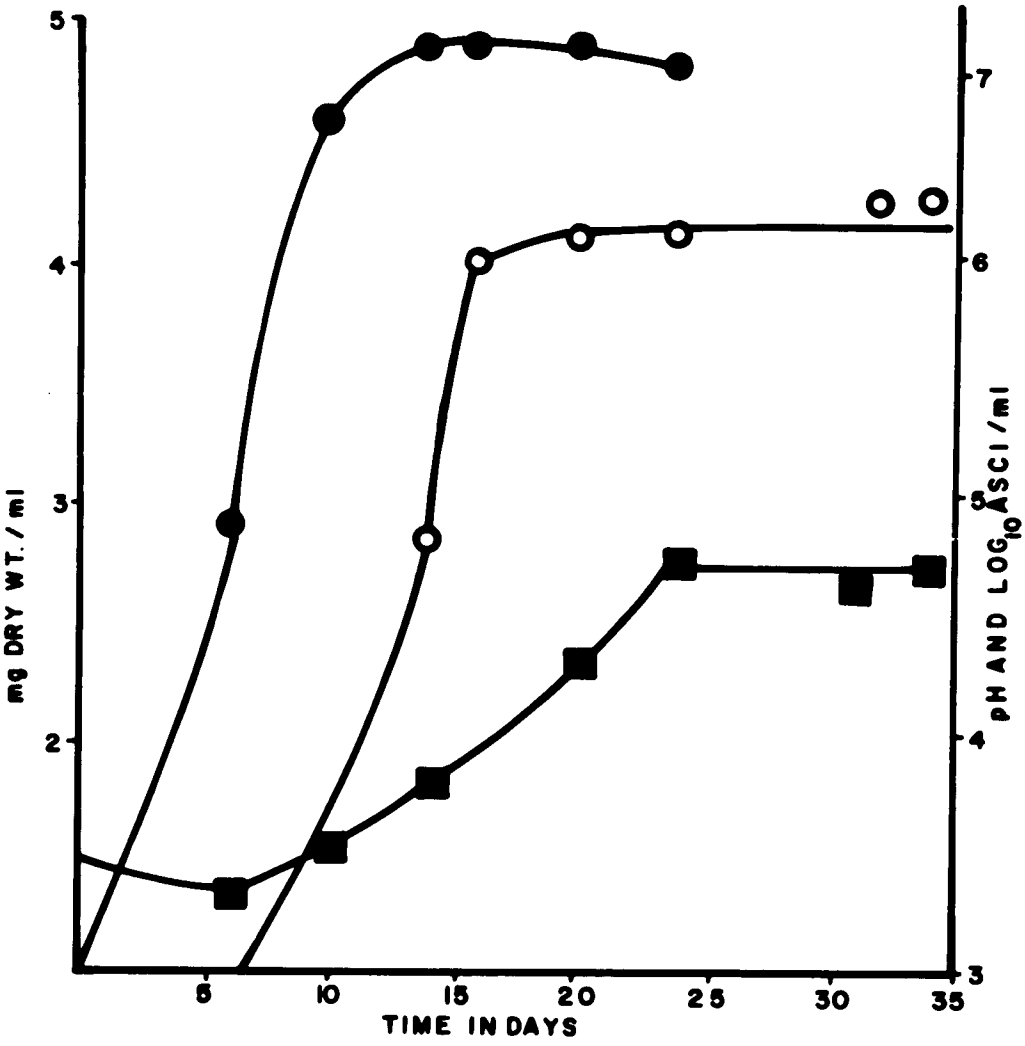
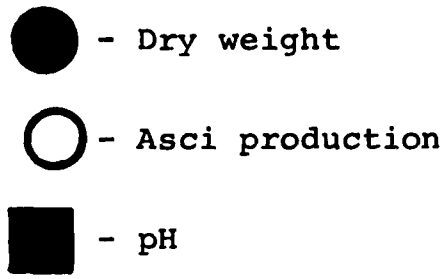
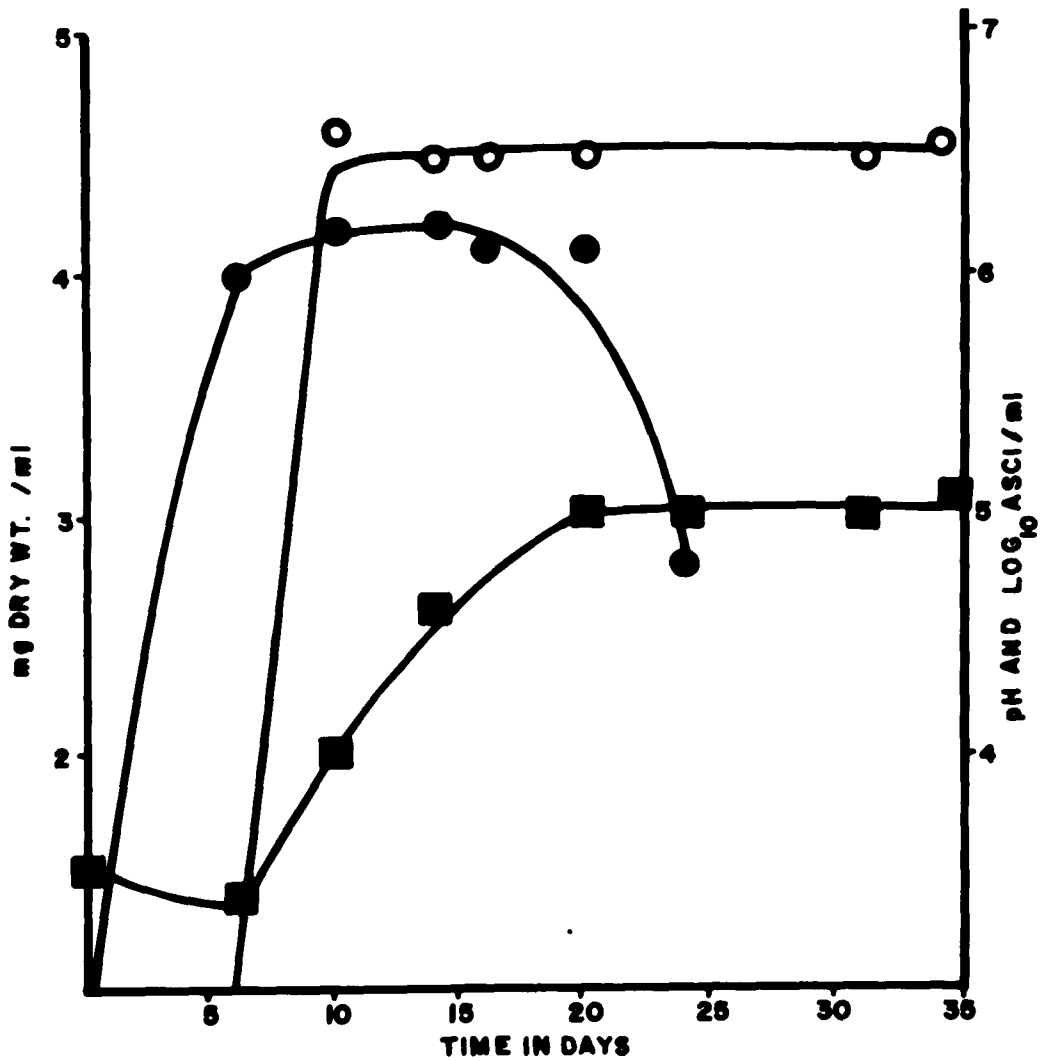

5
4
3
2
mg DRY WT. / ml
7
6
5
4
3
pH AND LOG10 ASCI / ml
5
10
15
20
25
30
35
TIME IN DAYS

Fig. 4. Dry wt., asci production and pH in non-synthetic medium composed of potato extract (0.5%) and 2% sucrose. Inoculum 50 conidia/ml.




5
4
3
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4
mg DRY WT. /ml
pH AND LOG10 ASCI/ml
5
10
15
20
25
30
35
TIME IN DAYS

shock appeared constant in the complete synthetic medium with sucrose from spore crop to spore crop and did not seem to vary over an extended period of time (Table 7).

IV. Preparation of purified asci suspensions

Fig. 5 shows a partially purified asci suspension containing conidia and hyphal strands. Fig. 6 shows a purified asci suspension containing less than 3.0% contamination with conidia and hyphal strands.

V. Fatty acid profiles of asci, conidia and hyphae

The fatty acid profiles and relative percent compostion of the different fatty acids present in asci, conidia, and hyphae are compared in Tables 8 and 8a. The asci contained a greater variety of fatty acids and a greater quantity of fatty acids than the hyphae or conidia. Also, the major portion of the saturated fatty acids of the asci are greater than 20 carbons in length while the greatest amount of the saturated fatty acids of the hyphae and conidia were between carbon chain lengths of C11 and C18. The major saturated fatty acids of the asci are br-C26, C27 and unknown #12.

Several fatty acids found in asci were not completely extracted at 25 C (Table 8), but were extracted efficiently at 56 C (Table 8a). Fatty acids exhibiting this behavior were C12, C14, C15, C16, C16:1, C17, C18, unknown #9

Table 7

Viable count of asci after heat shock for 2 hours in 0.01 M phosphate buffer (pH 7.0) from separate experiments.

Asci from synthetic medium*	Age of culture in days	Total count	Viable count	Viable count x 100 / Total count
	14	1.6×10^6	1.4×10^5	8.7
	16	4.0×10^5	5.4×10^4	13.5
	24	1.0×10^6	8.5×10^4	8.5
			1.1×10^5	11.0
	40	1.1×10^6	1.0×10^5	10.0
	40	2.5×10^6	2.9×10^5	11.6
Asci from potato dextrose broth	14	1.8×10^6	1.7×10^5	9.4
			1.6×10^5	8.8

* - Synthetic medium composed of Czapek's salts mixture, riboflavin (9.0 mg/l), nicotinamide (100 mg/l), ascorbate (200 mg/l), $CaCl_2$ (5.5 g/l) and 2% sucrose.

Fig. 5. Partially treated culture homogenate containing asci, conidia and hyphal fragments.

Fig. 6. Purified asci suspension.

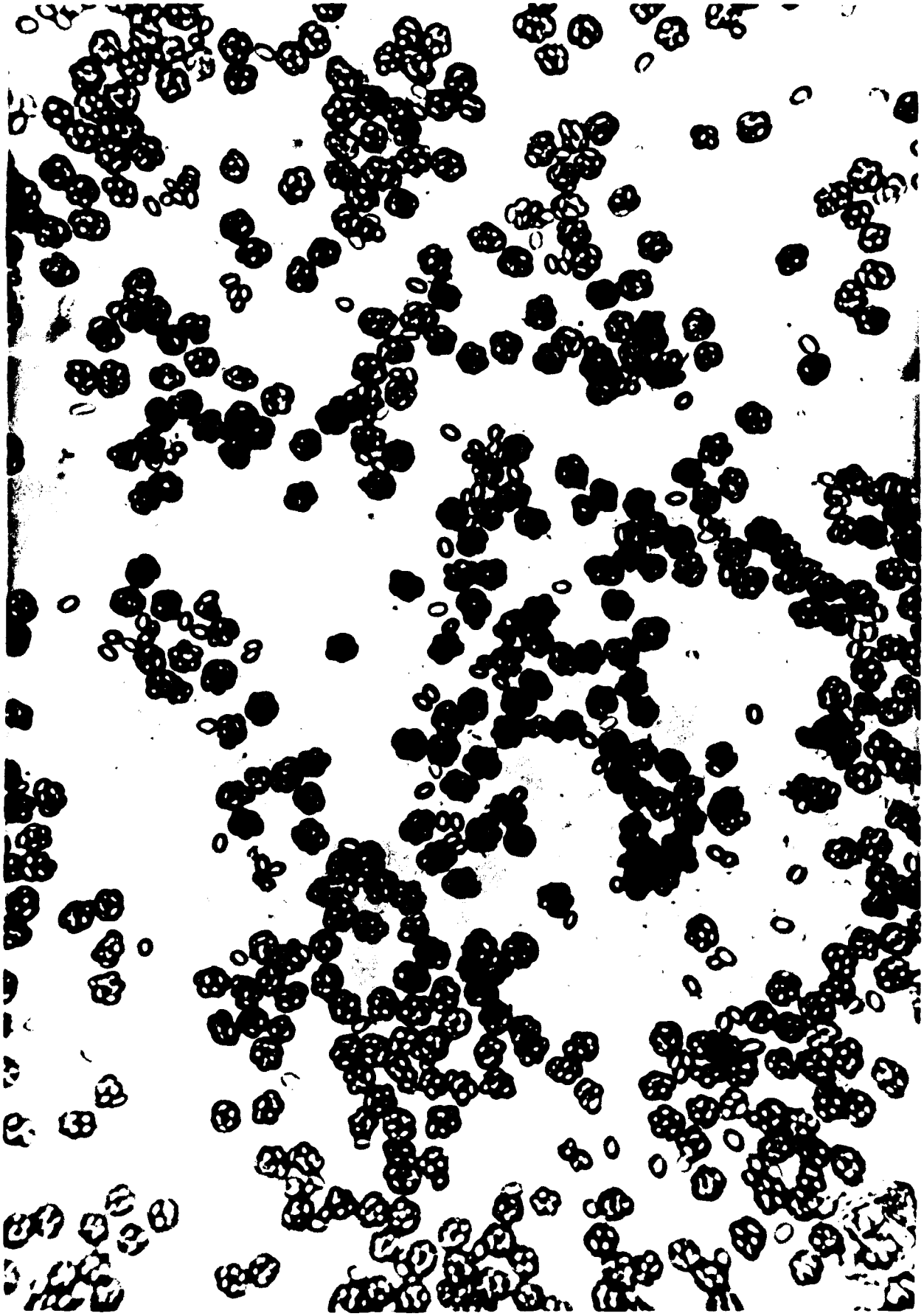


Table 8

Percent fatty acid composition of asci conidia and hyphae extracted at 25 C.

Fatty acid	Asci	Hyphae	Conidia
C11	1.40	1.60	1.20
C12	0.08	0.18	0.61
C13	0.28	0.36	0.83
C13:1			
C14	0.51	0.53	1.32
C14:1	0.25	0.45	0.41
C14:2			0.19
C15	0.16	0.55	1.54
C15:1		0.13	0.18
C15:2			0.07
C16	1.87	10.58	3.39
C16:1	0.89	1.00	0.61
C16:2	0.04	0.24	0.04
C17	0.08	0.28	0.47
C17:1		0.35	0.54
i-C18	0.17	0.59	0.04
C18	1.20	8.09	3.98
C18:1	27.96	17.82	46.00
C18:2	7.46	48.95	35.82
C18:3		0.32	0.08

Table 8 (continued)

Percent fatty acid composition of asci conidia and hyphae extracted at 25 C.

Fatty acid	Asci	Hyphae	Conidia
unk. (Sat'd)	0.06	0.39	0.47
unk. (Sat'd)	0.30		
unk. (Sat'd)	0.20	1.26	0.59
unk. (Sat'd)	0.16	0.09	0.37
C21	0.77	1.09	0.50
unk. (Sat'd)	0.92	0.54	0.48
C22	0.16	0.43	0.20
unk. (Sat'd)	0.15		
br-C23		0.28	
C23	0.17	0.22	
br-C24		1.66	1.11
unk. (Sat'd)		0.22	
unk. (Unsat'd)	0.25		
unk. (Sat'd)		0.31	
br-C26	5.77	0.69	
unk. (Sat'd)			
C27	22.96	0.12	
unk. (Sat'd)	2.80		
unk. (Sat'd)	17.04		

Table 8a

Percent fatty acid composition of asci, conidia and hyphae extracted at 56 C.

Fatty acid chain length	Asci	Hyphae	Conidia
C11	1.57		
C12	1.36		
C13	0.73		
C13:1	1.40		
C14	3.32	0.40	0.93
C14:1	1.60		0.31
C14:2	1.60		0.28
C15	1.50	0.25	0.79
C15:1	1.76		0.26
C15:2			0.17
C16	13.20	10.22	11.10
C16:1	3.17	0.70	1.95
C16:2	0.59		0.19
C17	1.02	0.35	1.30
C17:1	0.78	0.23	0.51
i-C18			
C18	4.99	13.17	12.26
C18:1	19.58	15.90	36.20
C18:2	7.92	49.06	24.97

Table 8a (continued)

Percent fatty acid composition of asci, conidia and hyphae extracted at 56 C.

Fatty acid chain length	Asci	Hyphae	Conidia
C18:3	0.85	0.08	0.16
unk. (Sat'd)	0.57	0.32	0.30
unk. (Sat'd)	0.57		0.13
unk. (Sat'd)	0.92	0.27	0.64
unk. (Sat'd)	0.36	0.88	0.53
C21	0.36	2.79	0.16
unk. (Sat'd)		0.36	2.01
C22	1.00		0.38
unk. (Sat'd)	0.58		0.09
br-C23			
C23			
br-C24		3.18	1.11
unk. (Sat'd)		0.22	0.44
unk. (Sat'd)	0.90		1.10
unk. (Sat'd)	2.47		
br-C26	3.44	1.00	0.74
unk. (Sat'd)	1.48		
C27	9.30		0.09
unk. (Sat'd)			

Table 8a (continued)

Percent fatty acid composition of asci, conidia and hyphae extracted at 56 C.

Fatty acid chain length	Asci	Hyphae	Conidia
unk. (Sat'd)	6.65		

and unknown #10. However, fatty acids C18:1, C27, unknown #11 and unknown #12 were nearly completely extracted at 25 C.

The fatty acids i-C20, unknown #1, unknown #2, C21, unknown #3, C22, unknown #4, br-C23, unknown #5, unknown #6, br-C24, unknown #7, and unknown #8, were found only in trace amounts (less than 1 relative percent) after extraction at 25 C and reflux temperature (56 C).

After extraction for 4 days at 25 C, microscopic examination of the asci (ascospore) preparations showed almost complete disintegration of the asci to yield individual ascospores. Very few non-refractile ascospores were noted although some internal structure could be noted in many.

Media inoculated with asci (ascospores) after each extraction showed that some of the spores were still viable. Media inoculated with conidia extracted for 4 days at 25 C showed no growth. Hyphae were not tested for viability after extraction.

VI. Analysis of the ascospores

A. Determination of percent spore coat per ascospore

The results reported in Table 9 indicate that approximately 60% of the ascospore consists of spore coat on a dry weight basis.

B. Lipid composition of the ascospore, ascospore coat and cytoplasm

Based on the dry weights recorded in Table 9, 22.75% of the ascospore consists of lipid while the lipid content of the ascospore coat and cytoplasm is 10.50% and 42.73% respectively. Most of the lipid was found in the sporoplasm.

C. Lipid classes

The lipid classes identified in ascospore cytoplasm and ascospore coats are listed in Table 10.

No sterol or sterol esters were detectable under the procedures employed. Unknown #1 was a fluorescent material which required no spraying for detection.

Based on band width and intensity of color, more hydrocarbon and diglyceride was noted in the sporoplasm than in the ascospore coat. Initially unknown #2 appeared to be present in a larger quantity in the sporoplasm, but the fluorescence diminished on standing for 24 hours at which time the unknown appeared to be present in the spore coats in greater quantity. There appeared to be more of unknown #1 in the ascospore coats than in the sporoplasm. Triglycerides, free fatty acids, and phospholipids showed an equal distribution between spore coats and sporoplasm.

D. Quantitation of fatty acids in the sporoplasm and ascospore coat fractions

Dry weight values in percentages for sporoplasm and ascospore coats are recorded in Tables 11 and 12. Fatty acids in the sporoplasm amounted to 8.37% of the sporoplasm

Table 9

Dry weights and percentages of lipid in ascospore, ascospore coat and sporoplasm; percent ascospore composition as ascospore coat and sporoplasm.

Structure	Dry wt. in mg	Lipid Extracted in mg	% lipid	Percent composition of ascospore	% of the total lipid
Ascospore	415.15	93.45	22.50		99.94
Ascospore coat	254.20	25.45	10.50	61.23	73.00
sporoplasm*	160.95	69.00	42.73	38.76	26.94

* - Weight of intact asci minus weight of ascospore coat.

Table 10

Lipid classes present in the sporoplasm and ascospore coats in the order of their relative positions on TLC plates.

HYDROCARBON

UNKNOWN #2

TRIGLYCERIDES

FREE FATTY ACIDS

DIGLYCERIDES

UNKNOWN #1

MONOGLYCERIDES

PHOSPHOLIPIDS

Direction of solvent development.

Table 11

Percent fatty acid composition of sporoplasm based on dry weight determinations.

Fatty acid chain length	Micrograms dry weight	Percent composition of total fatty acids
C11	51.73	0.89
C12	43.29	0.75
C13	324.90	5.62
C13:1	7.96	0.13
C14	97.89	1.69
C14:1	29.71	0.51
C14:2	7.96	0.13
C15	15.51	0.26
C15:1	46.42	0.80
C16	835.66	14.47
C16:1	77.70	1.34
C16:2	13.32	0.23
C17	33.42	0.57
C17:1	36.61	0.68
i-C18	57.83	1.00
C18	378.30	6.55
C18:1	1431.21	24.73
C18:2	419.16	7.26
C18:3	3.97	0.06

Table 11 (continued)

Percent fatty acid composition of sporoplasm based on dry weight determinations.

Fatty acid chain length	Micrograms dry weight	Percent composition of total fatty acids
C20	46.20	0.80
unk. #1	58.10	1.00
unk. #2	29.85	0.51
C21	11.35	0.13
unk. #3	189.02	3.27
C22	65.37	1.13
unk. #4	4.16	0.07
br-C23	30.64	0.53
C23	150.95	2.61
unk. #5	350.98	6.08
unk. #5	20.72	0.35
br-C24	46.69	0.80
unk. #7	3.18	0.05
unk. #8*	382.00	6.61
unk. #8	2.92	0.05
br-C26	64.62	1.10
unk. #10	55.70	0.96
C27	239.30	4.14
unk. #11		

Table 11 (continued)

Percent fatty acid composition of sporosplasm based on dry weight determinations.

Fatty acid chain length	Micrograms dry weight	Percent composition of total fatty acids
unk. #12	201.62	3.49

* - The only known unsaturated fatty acid with a carbon chain length greater than 19.

Table 12

Percent fatty acid composition of ascospore spore coat based on dry weight determinations.

Fatty acid chain length	Micrograms dry weight	Percent composition of total fatty acids
C11	13.94	0.35
C12	5.00	0.12
C13	75.53	1.92
C13:1		
C14	20.44	0.52
C14:1	8.89	0.22
C14:2	15.83	0.40
C15	5.64	0.14
C15:1	9.55	0.24
C16	122.74	3.13
C16:1	7.43	0.18
C16:2	1.06	0.02
C17	9.03	0.23
C17:1	10.60	0.27
i-C18	14.44	0.36
C18	87.48	2.23
C18:1	1204.38	30.75
C18:2	495.69	12.65
C18:3	1.19	0.03

Table 12 (continued)

Percent fatty acid composition of ascospore spore coat based on dry weight determinations.

Fatty acid chain length	Micrograms dry weight	Percent composition of total fatty acids
C20	14.94	0.38
unk. #1	8.00	0.20
unk. #2	4.28	0.10
C21	16.19	0.41
unk. #3	33.88	0.86
C22	21.99	0.56
unk. #4		
br-C23	14.88	0.37
C23	26.38	0.67
unk. #5	69.42	1.77
unk. #6		
br-C24	154.12	3.93
unk. #7		
unk. #8*	102.19	2.60
unk. #9	21.94	0.56
br-C26	202.70	5.18
unk. #10	41.10	1.04
C27	715.35	18.26
unk. #11	27.21	8.70

Table 12 (continued)

Percent fatty acid composition of ascospore spore coat based on dry weight determinations.

Fatty acid chain length	Micrograms dry weight	Percent composition of total fatty acids
unk. #12	478.75	12.22

* - The only known unsaturated fatty acid with a carbon chain length greater than 19.

lipid while the fatty acids of the ascospore coats accounted for 15.39% of the ascospore coat lipid.

The C11 through C18 saturated fatty acids, which constitute 40.36% of the total saturated fatty acids of the ascospore, are found mainly in the sporoplasm.

Approximately 59.64% of the total saturated fatty acids of ascospores have chain lengths greater than 19 carbons. Of this long chain group, fatty acids unknown #3, br-C24 unknown #10, C27, unknown #12, and unknown # 13, predominated in the ascospore coats, while the remainder appeared mainly in the sporoplasm.

The saturated fatty acids did not follow the distribution pattern of the more commonly membrane-associated fatty acids (C18:1 and C18:2) which exhibited an almost equal distribution between the ascospore coat fraction and the sporoplasm fraction.

VII. Fatty acid content of the lipid classes from the sporoplasm and ascospore coat fractions

A. Phospholipids (Table 13). This class differs from the other classes in that it has the largest concentrations of C23 and unknown #8. The former fatty acid is found mainly in the sporoplasm while the latter was nearly equally distributed between spore coat and sporoplasm. Fatty acids C27 and unknown #12 were found mainly in the ascospore coat, while C16 and C18 were found in the highest

concentrations in the sporoplasm. The fatty acids of the sporoplasm and ascospore coats were approximately 60% saturated.

B. Triglycerides (Table 14). The fatty acids in this class showed two salient features which distinguished it from the other classes: (1) the sporoplasm i-C18 content is 37.10% and the coat content is 7.13% and (2) it has an unusually high percentage of C13 in the ascospore coat fraction. Both fractions also contained an unknown immediately following br-C26 in retention time which no other class of lipid possessed. The triglycerides seemed to lack significant amounts of unknown #11 and unknown #12. The fatty acids in the sporoplasm were 68% saturated and the fatty acids of the ascospore coat fraction were 77% saturated.

C. Unknown #2 (Table 15). This class had the highest percentage composition of C18:2, C27 and unknown #12. Fatty acids C27 and unknown #12 were located mainly in the ascospore coat while C18:2 was located mainly in the sporoplasm. Forty-three percent of the sporoplasm fatty acids were saturated while the coat fraction showed 66% saturation.

D. Diglycerides (Table 16). This class differs from all of the other classes by one salient feature. This class in both fractions, possessed the lowest relative concentrations of the C18 series of fatty acids. The major portion of the fatty acids of the sporoplasm consisted of C16

Table 14

Percent fatty acid composition of triglycerides from sporoplasm and ascospore coats

Fatty acid chain length	Sporo-plasm	Spore coat	Fatty acid chain length	Sporo-plasm	Spore coat
C11		9.80	i-C20		1.00
C12		4.65	C20	0.37	0.90
C13	0.71	16.91	unk. #1	0.31	
C13:1	0.13		unk. #2	0.16	
C14	1.23	2.76	C21	0.76	2.32
C14:2	0.49		unk. #3		0.23
C15	1.23	1.09	br-C23	0.59	2.09
C16	7.10	7.02	unk. #5	3.63	0.56
C16:1	1.94	1.09	unk. #6	0.49	0.50
C16:2	0.49	2.00	br-C24	0.37	0.48
C17	0.63	1.46	unk. #7	0.50	0.63
C17:1	0.58	1.44	unk. #8*	0.82	0.63
i-C18	37.10	7.13	unk. #9	1.75	2.21
C18	2.26	4.08	br-C26	2.06	1.74
C18:1	17.64	12.82	unk.**	4.34	5.98
C18:2	9.30	4.07	unk. #10	0.76	1.61
C18:3	0.26		C27	1.49	1.96

* - The only known unsaturated fatty acid with a carbon chain length greater than 19.
**- Fatty acid not detected until this class was selectively examined: saturation level not determined.

Table 15

Percent fatty acid composition
Unknown #2 from sporoplasm and ascospore coats

Fatty acid chain length	Sporo-plasm	Spore coat	Fatty acid chain length	Sporo-plasm	Spore coat
C11		1.42	C20	0.66	0.33
C12	1.70	0.72	unk. #1	0.23	0.25
C13		1.10	unk. #2	0.43	0.30
C14	1.30	0.68	C21	0.63	0.27
C14:1	1.80		br-C23	0.46	0.62
C14:2		0.95	C23		0.28
C15	1.50	0.53	unk. #5		0.34
C15:1	1.80	0.79	unk. #6	0.59	0.44
C16	3.80	2.24	br-C24	0.38	2.83
C16:1	1.35	0.71	unk. #7	0.59	
C16:2	0.80	1.45	unk. #8*	0.09	1.79
C17	0.45	0.24	unk. #9	3.26	0.80
C17:1	0.36	0.25	br-C26	2.10	6.27
i-C18	0.36	0.24	unk.**		0.66
C18	2.40	1.71	unk. #10		0.32
C18:1	29.59	20.82	C27	14.64	21.01
C18:2	12.11	6.65	unk. #11		0.71
C18:3	0.46	0.15	unk. #12	8.03	21.36

* - refer to Table 14 page 62 for legend.
** - refer to Table 14 page 62 for legend.

Table 16

Percent fatty acid composition of diglycerides from sporoplasm and ascospore coats

Fatty acid chain length	Sporo-plasm	Spore coat	Fatty acid chain length	Sporo-plasm	Spore coat
C11		5.07	unk. #1	0.60	
C12		2.91	C21	0.10	0.54
C13		3.76	unk. #3	0.10	0.32
C14	0.10	4.74	br-C23	19.50	5.42
C14:1		6.42	C23	2.60	4.50
C14:2		6.99	br-C24	0.40	
C15	1.20	5.48	unk.**	0.20	
C15:1		7.55	unk. #7	55.70	
C16	4.30	5.32	unk. #8*	4.00	12.05
C16:1	0.50	3.04	unk. #9		1.18
C16:2	0.30		unk.**		1.23
C17	0.10	1.82	unk.**		0.63
i-C18	0.10		br-C26	0.60	1.31
C18	2.00	2.58	unk. #10	0.80	2.03
C18:1	2.00	3.33	unk.**		1.14
C18:2	0.80	1.56	unk.**		1.25
i-C20		0.58	C27		3.22
C20	2.10		unk. #12		1.64

* - refer to Table 14 page 62 for legend.
**- refer to Table 14 page 62 for legend.

(4.3%), br-C23 (19.5%), and unknown #7 (55.7%). The saturated fatty acids accounted for 91.3% and 55.22% of the fatty acids of the sporoplasm and ascospore coats respectively. Of the fatty acids in the ascospore coat fraction, no one fatty acid exceeds a relative concentration of 6.0%. The ascospore coat fraction also contains C27 and unknown #12 which were not detected in the sporoplasm and also possessed a higher relative percent of unknown #8 than the diglycerides from the sporoplasm.

E. Free fatty acids and monoglycerides (Tables 17 and 18).

These two classes closely resembled one another in fatty acid composition. They both lacked unknown #11 and unknown #12, a characteristic they share with the triglycerides and fluorescent unknown. Both classes possessed about the same relative percentages of C16 and C18 whether or not they were found in the ascospore coats or sporoplasm. One feature these two classes have in common, but which is not possessed by any of the other classes is the presence of two new unknowns immediately following unknown #10 in retention time.

The free fatty acids were 69% saturated in the sporoplasm and 56.94% saturated in the ascospore coat fraction.

F. Fluorescent Unknown (Unknown #1, Table 19). In this class approximately 86% of the fatty acids are in the C14 to c18:2 range. It is the only class which fluoresces

Table 17

Percent fatty acid composition of
free fatty acids from sporoplasm and ascospore coats

Fatty acid chain length	Sporo-plasm	Spore coat	Fatty acid chain length	Sporo-plasm	Spore coat
C14		13.22	unk. #1	0.70	0.27
C14:1		7.67	unk. #2	0.10	0.47
C15	2.70	7.42	C21	4.70	0.90
C16	24.20	11.61	br-C24	1.10	1.55
C16:1	5.80	4.45	unk. #7	0.60	0.60
C16:2		2.26	unk. #8*	0.30	
C17	1.60		unk. #9	3.30	5.30
C17:1	1.30		br-C26	0.90	2.05
C18	18.50	5.05	unk.**	0.50	
C18:1	19.00	13.78	unk. #10	1.60	1.27
C18:2	2.80	12.72	unk.**	1.20	0.48
C18:3	0.40		unk.**	1.20	1.17
i-C20	0.01		C27	1.40	2.65
C20	1.70	0.60			

* - refer to Table 14 page 62 for legend.
**- refer to Table 14 page 62 for legend.

Table 18

Precent fatty acid composition of monoglycerides from the sporoplasm and ascospore coats

Fatty acid chain length	Sporo-plasm	Spore coat	Fatty acid chain length	Sporo-plasm	Spore coat
C14	3.64	11.20	unk. #2	0.25	
C14:1	2.92	5.14	C21	0.11	0.91
C14:2	1.19		unk, #3	0.25	0.63
C15	1.91	3.29	unk. #4		0.67
C15:1	1.53	4.21	br-C23	0.67	1.02
C16	24.00	15.09	C23	0.10	
C16:1	4.11	7.71	unk. #5	0.86	1.07
C16:2	0.34	3.53	br-C24	0.43	
C17	0.65	1.84	unk. #7	1.14	1.46
C17:1	0.74		unk. #8*	0.31	1.24
C18	8.23	6.54	unk. #9	0.52	0.93
C18:1	31.93	18.99	br-C26	4.49	3.16
C18:2	3.86	3.07	unk. #10	1.66	2.65
i-C20	0.60		unk. **	0.23	0.92
C20	0.67	0.20	unk. **	0.39	1.61
unk. #1	0.34		C27	0.46	2.92

* - refer to Table 14 page 62 for legend.
**- refer to Table 14 page 62 for legend.

Table 19

Percent fatty acid composition of the fluorescent unknown; coats and supernatant were pooled

Fatty acid chain length	Percent concentration
C14	13.38
C16	19.88
C16:1	7.97
C18	8.36
C18:1	31.01
C18:2	6.39
br-C23	1.62
unk. #6	1.57
br-C24	0.94
unk. #7	1.39
unk. #9	1.70
unk.**	2.67
unk. #10	3.05

** - refer to Table 14 page 62 for legend.

and absorbs in ultraviolet light.

VIII. Electron microscopy of ascospores and conidia

A. Conidia (Fig. 7) One may note in Fig. 7 a thin section of a B. fulva conidium which is representative of the sections examined. The conidia possess a well developed highly involuted cytoplasmic membrane and many mitochondria. The thin section represented here also displays a plasmalemmasone-like structure which appears to be an infolding of the inner unit membrane. This was the only section found which displayed such membranous infoldings. All conidial thin sections showed easily recognizable inner and outer spore coats. Although not reported here, many thin sections showed well developed nuclei with nuclear pores.

B. Ascospores (Fig. 8 through 12). One may note in Fig. 8 through 11 that the remnant of the ascus membrane is distinct and surrounds the individual ascospores in a manner similar to a bacterial spore exosporium. The ascus membrane seems to lack unit membrane structure and resembles a limiting membrane.

A cytoplasmic membrane is present in all thin sections but appears in two forms depending on the structural differentiation of the ascospore. Fig. 8 shows one type of cytoplasmic membrane structure which displays some involutions but not to the degree seen in conidia. Fig. 9

Fig. 7. Thin section of a B. fulva conidium. M - mitochondrion, ib - inclusion body pm - plasmalemmasome-like, ic - inner coat, oc - outer coat, cm - cytoplasmic membrane, cmi - cytoplasmic membrane invaginations.

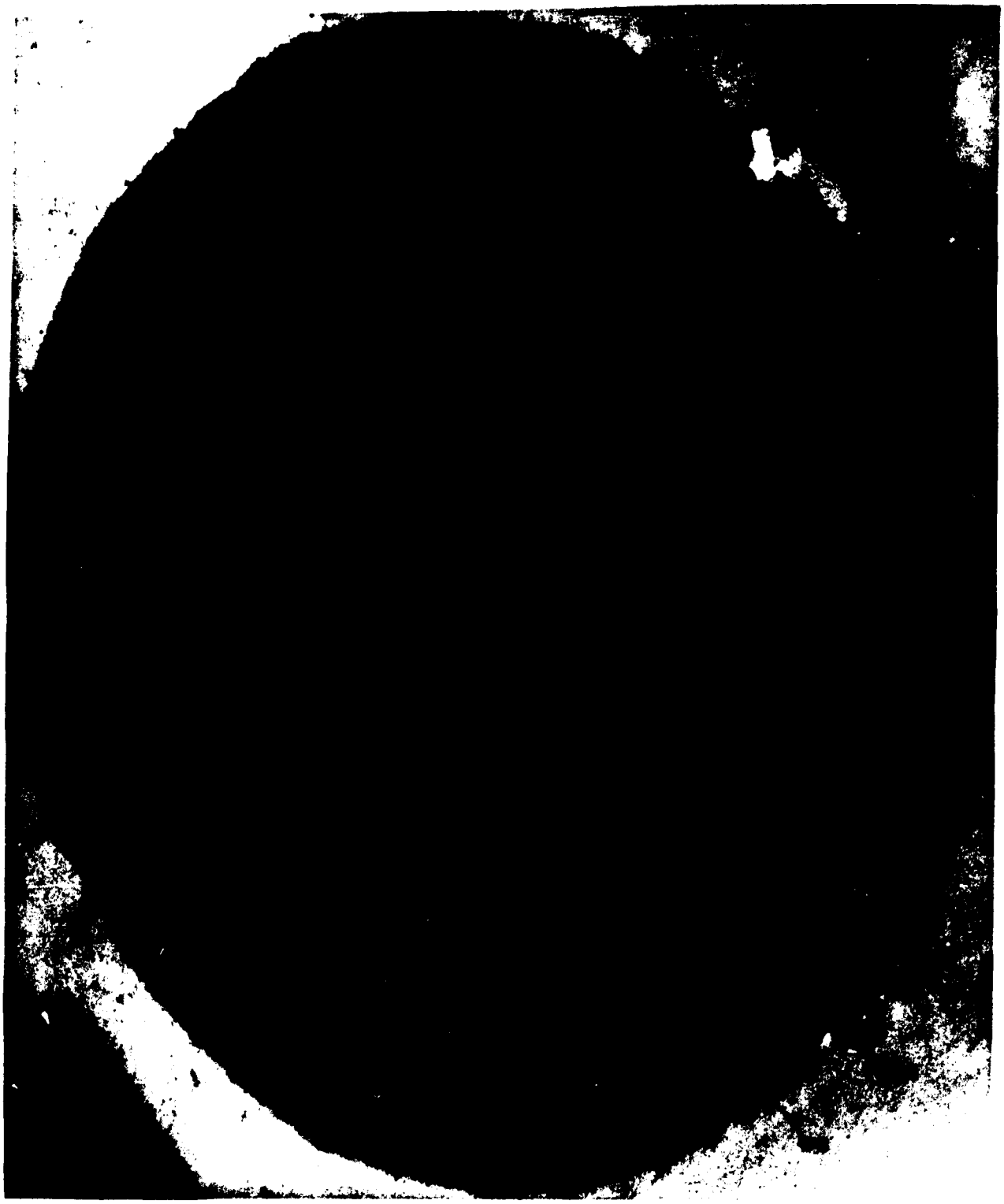


Fig. 8. Longitudinal section through a B. fulva ascospore. am - ascus membrane, ic - inner coat, oc - outer coat, cm - cytoplasmic membrane, M - mitochondrion, N - nucleus, nm - nuclear membrane.

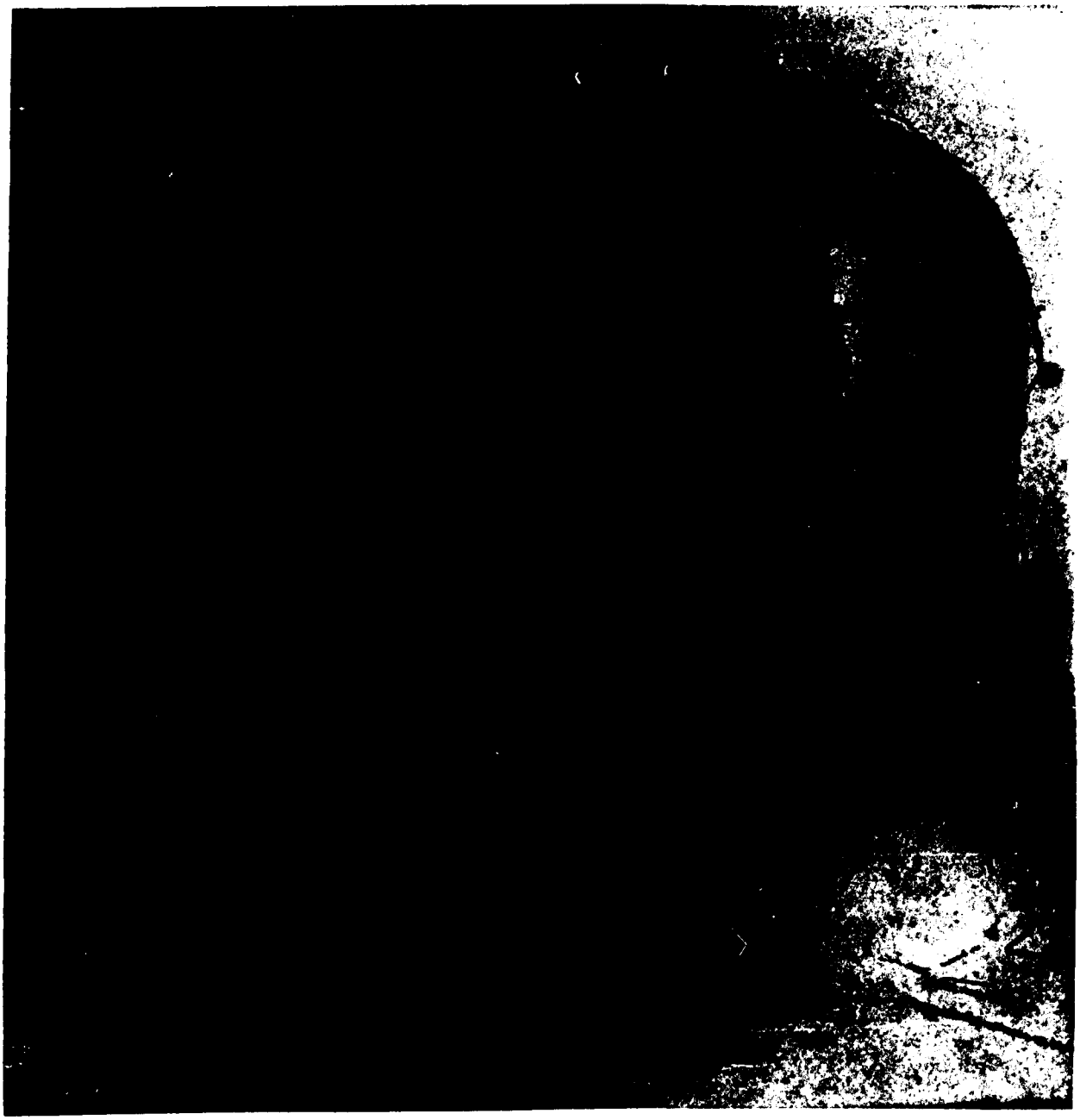


Fig. 9. Longitudinal section through a B. fulva ascospore. am - ascus membrane, ib - inclusion body, ic - inner coat, oc - outer coat, nm - nuclear membrane.

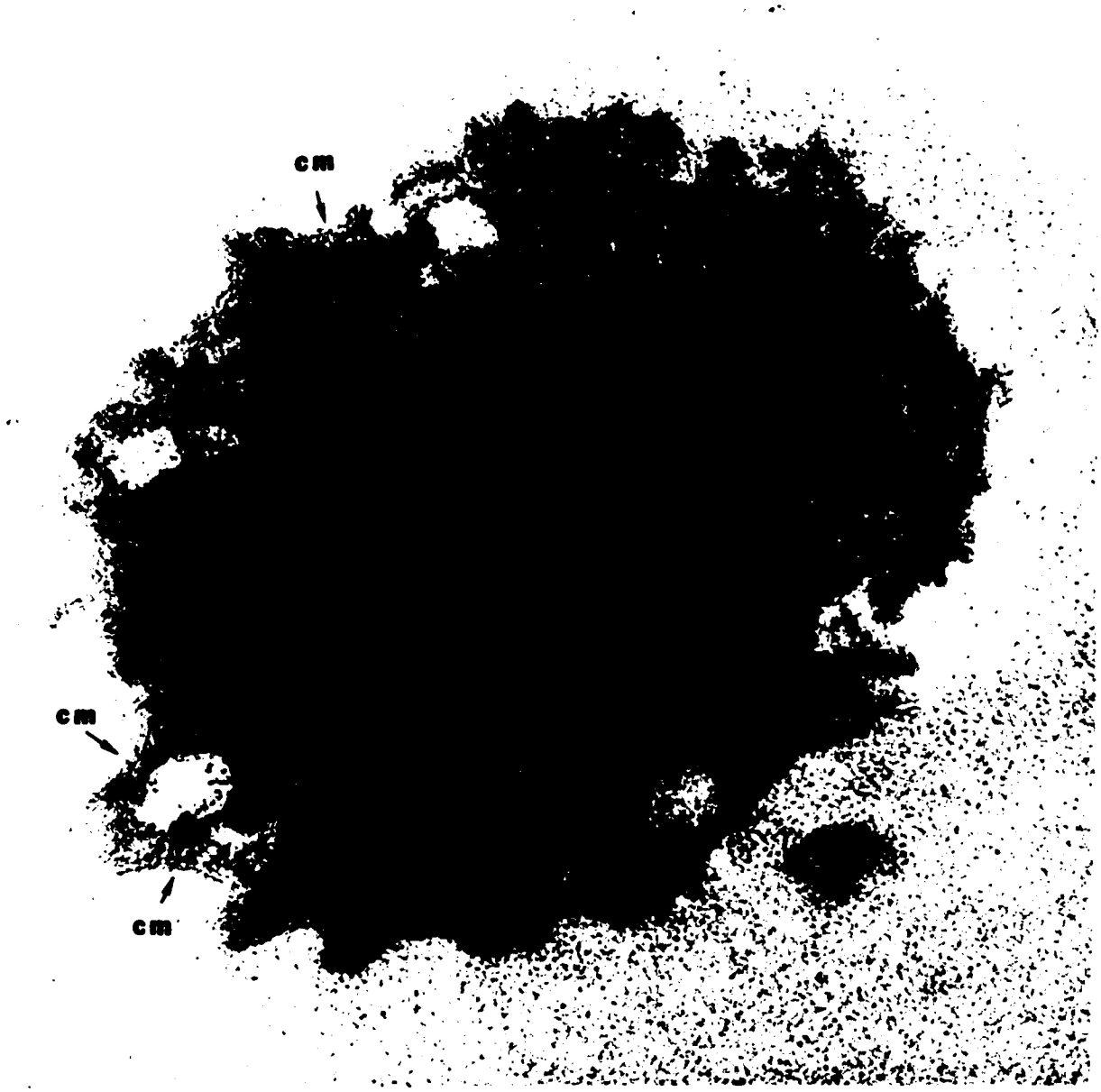

oc
am

Fig. 10. Cross-section through a B. fulva ascospore. cm - cytoplasmic membrane, ib - inclusion body, ic - inner coat, oc - outer coat.

Fig. 11. Cross-section through a B. fulva ascospore. am - ascus membrane, oc - outer coat, ic - inner coat cm - cytoplamic membrane, ib - inclusion body.


a.m
c.m

Fig. 12. Enlargement of the sporoplast shown in Fig. 11. For legend see Fig. 11.


cm
cm
cm

through 12 show the second type of cytoplasmic membrane structure. This type exhibits a highly involuted membrane where the membrane seems to be pulled together or compressed. This type of structure is best seen in Fig. 12.

The ascospores exhibit a thick spore coat which can be readily broken down into an inner and outer spore coat.

Mitochondria are evident only in Fig. 8 where several can be seen.

A nuclear membrane is evident in Fig. 8 and 9. The other figures seem to be lacking a nuclear membrane which is probably due to the dark staining material.

Cytoplasmic inclusion bodies which stain very much like the inner spore coat material may be noted in Fig. 9 through 12. These inclusion bodies are lacking in the structurally differentiated ascospore (Fig. 8).

DISCUSSION

The genus Byssochlamys, until the last few years, has been considered a laboratory curiosity. However, its growing involvement in food spoilage (Maunder, 1969) has quite suddenly placed it in the mainstream of microbial research.

The first experiments reported in the present work were concerned with the isolation of single ascospores (Tables 1 and 2). The observations of the cultures produced upon germination of the ascospores have established two basic facts about Byssochlamys fulva (National Canners Association strain 68-10) which have not been previously reported: (1) the strain of B. fulva is homothallic, since the cultures resulting from the out-growth of a single ascospore will form 8-spored asci and (2) the majority of the single ascospore isolates do not resemble the parent culture in pigmentation, growth rate or quantitative asci production.

The isolated strains could be readily placed into four groups by utilizing a synthetic agar medium (Table 3, Experiments III and IV). One group (Pattern 1 page 25) exhibited white to off white growth with good asci production after 12 to 16 days and corresponded to Brown and Smith's (1957) description of Byssochlamys nivea. A sec- group (Pattern 2) exhibited a buff type of growth and good

asci production after 12 to 16 days and corresponded to Brown and Smith's description of Byssochlamys fulva. The remaining two groups (Patterns 1a and 2a page 25) appeared to be variants of the B. nivea- and B. fulva-like strains. The variant of the B. nivea-like strain originally produced white mycelia which upon continued incubation produced a reddish pigment and low numbers of asci. The variant of the B. fulva-like strain produced a buff mycelium initially, but with continued incubation it produced a reddish-brown pigment and low numbers of asci. The poor asci production and the reddish-brown pigment are atypical for B. fulva and B. nivea. Strain variation is well known in B. fulva especially in regard to asci production (Denny and Brown, 1969; Maunder, 1969; Prest, 1969). The importance of standardized cultural procedures for characterization of various strains has been stressed (Prest, 1969). Potato dextrose agar, the most common plating medium for B. fulva, was not a good medium for delineating the characteristics which are used for identifying strains (Table 3, Experiments I and II, Table 4). The establishment of clear, easily recognizable cultural patterns upon repeated sub-culturing indicates the synthetic agar medium may be valuable as a tool for studying strain variation in Byssochlamys sp. Another synthetic medium commonly used for separating B. fulva from B. nivea

is Czapek's agar (Brown and Smith, 1957). However, Czapek's has not been used for studying strain variation. Our own observations have shown that Czapek's agar or liquid media does not support asci production as well as does potato dextrose agar or the synthetic liquid media (Medium A Fig. 1).

Of the 34 isolates examined, 18.91% resembled the parent culture in asci production and colonial morphology (Pattern 2 Table 3, Experiments III and IV; Table 4). Thirty-two percent resembled B. nivea, while 21.62% and 27% were B. nivea-like and B. fulva-like variants respectively. The low value for the parental phenotype was unexpected. The results indicate a genetically diverse organism.

The ascospores not only responded differently to an external stimulus in the number of ascospores which could be induced to germinate, but also responded differently in the time of heating required to induce germination (Table 2). This seems to be an excellent mechanism for insuring the survival of the organism. At any one time, even if the conditions seem favorable, all of the ascospores will not germinate insuring that some of the ascospores will be available for germination at a later date. This timing of germination over an extended period of time would increase the organism's chances of coming into contact with

an environment conducive to ascospore formation.

Since the ascospores were picked in a random manner, probability favors the ascospores originating from different asci rather than any originating from the same ascus. Although it has not been shown experimentally, the results indicate that clones of asci are being produced. It is indeed possible that the white granules described in the literature (Brown and Smith, 1957) may represent genetically homologous clones of asci. Probability also favors each ascospore coming from a separate granule. Experimentation would be required to prove or disprove this hypothesis. If genetically homologous clones were produced, the tools and experience of basic bacteriology could be applied to the study of B. fulva since each clone could be treated as a colony. Our own observations have shown that the white granules do contain masses of asci. The granules possess a gelatinous consistency and are segregated from one another so that they may be easily picked with the aid of a dissecting microscope and a fine needle.

The B. nivea-like and B. fulva-like strains, the asci of which exhibited high percentages of germination upon heat shock, may prove valuable in heat inactivation studies, because heat inactivation curves are difficult to interpret unless 100% of the asci or ascospores can be accounted for in terms of germination and death. No experiments were carried out with single ascospore suspensions

of these strains and extrapolation of results obtained with the asci to ascospores does not seem valid. However, these variants do give the highest heat shock induced germination percentages of any of the strains tested and these high values have not been previously reported for B. fulva.

The experiments involved with ascospore dormancy within the ascus also yielded unexpected results. When a suspension of single ascospores from strain Bf-25-10 was heated at 70 C for two hours and plated out on PDA, only 3.45% of the ascospores from a 14 day old culture and 7.5% of the ascospores from a 40 day culture could be induced to germinate (Table 6). However, if one considers the 10% heat induced germination of asci shown by strain Bf-25-10 at 70 C (Table 5), then at a maximum only 10% of the individual ascospores should be susceptible to heat shock induced germination under the same conditions if all of the ascospores within a heat shockable ascus could be induced to germinate. This 10% should have been true with the 14 day old and 40 day old cultures since 10% heat induced germination of intact asci was obtained(Table 7). A 100% germination of ascospores from the heat-activatable asci is evidently not the case. If one takes maturation to mean the ability of the ascospore to respond to heat shock by germination, the results in Table 6 indicate an asynchronous maturation of ascospores with time since the

number of ascospores/ascus which can be induced to germinate increases with time. Based on ascospore numbers/ascus, it seems that 2 ascospores/ascus can be induced to germinate in asci from a 14 day old culture and 6 ascospores/ascus will be capable of germination by heat shock of asci from a 40 day old culture. Asynchronous maturation of ascospores within the ascus of Hansenula anomala has been observed (Bandoni et al., 1966) and this phenomenon appears to be a characteristic of B. fulva.

Ascospores from a 40 day old culture of Bf-19-10, a B. nivea-like strain, were examined to determine if asynchronous maturation of ascospores occurred. If maturation similar to that observed with Bf-25-10 occurred, then 50% of the ascospores should have been susceptible to heat shock germination since heat shock experiments with intact asci revealed that approximately 50% of the asci can be induced to germinate upon heat shock (Table 5). However, the results in Table 6 show that only 6.8% of the ascospores (based on total ascospore count) could be induced to germinate by heat shock. If germination was based on 50% of the ascospores, a germination value of 13.6% is obtained which would account for 1 ascospore/ascus germinating. The results indicate three possibilities: (1) all of the ascospores of a heat activatable ascus are not viable, (2) all of the ascospores within a heat activatable ascus are viable but only 1 ascospore can be in-

duced to germinate, and (3) 91% of the total ascospore population was heat inactivated.

In regard to the series of heat shock germination experiments with separated ascospore, it may be that upon breakage of the asci with glass beads, a high percentage of the ascospores were killed or became heat sensitive. If this were the case, then 95% to 99% of the ascospores of the B. fulva-like strain and 99% of the ascospores of the B. nivea-like strain would have to be affected in one or both ways. However, this procedure has been previously used to break asci of B. nivea to obtain single ascospore suspensions and the results obtained do not indicate heat sensitivity or inactivation due to the treatment (Yates et al., 1968).

The low germination values for B. nivea and B. fulva ascospore may merely be due to lack of a good germination trigger. These germination triggers are known to play an important role in fungal spores (Sussman and Halvorson, 1966). This major handicap in studying Byssochlamys ascospores could possibly be removed by the isolation of strains which showed 100% germination of asci and ascospores (with or without inducers). These isolation would require a synthetic differential medium for rapid screening of strains and the establishment of basic parameters which could be easily recognized by someone not well versed in the intricacies of fungal morphology.

Preliminary experiments revealed that carbohydrate, riboflavin, nicotinamide, ascorbic acid and $CaCl_2$ were required additives to Czapek's basal salts in order to obtain good asci production. However, if any one or all of these ingredients except the sugar were omitted the cultures were not entirely lacking in asci. Heat shocking (70 C for two hours, pH 7.0) revealed that heat-resistant bodies were present, but at less than 1% of that obtained when calcium and vitamins were added.

The results recorded in Fig. 1 reveal that none of the synthetic media supported asci formation to the same extent of that observed with PDB and PSB. Peak asci formation was observed in 14 days with PDB and PSB whereas in the synthetic media the peak was reached in 16 to 20 days. Asci production in synthetic media on a dry weight basis ranged from 30% to 50% of that obtained with PSB and PDB. After maximum asci formation was attained, there was a decrease in asci counts with PDB but no decrease was noted with PSB and in synthetic media containing sucrose as the main carbon source during the time of the experiment. If glucose was employed as the main carbon source in the synthetic media, asci formation was erratic. Although it has not been shown experimentally, the asci in glucose-containing media may have germinated which would account for the decrease in asci with time. Whatever accounts for decreased asci numbers with time in glucose-containing

media, it is apparent that sucrose is the sugar of choice because the asci count in the sucrose-containing medium was as high as in the presence of glucose, and the asci count in the presence of sucrose remained constant over an extended period of time. A decrease in ascospore counts with time has been reported in complex media by Splittstoesser et al. (1969). The data in Fig. 1 also show no appreciable difference in asci production on synthetic media when the inoculum was increased by a factor of 10 or the calcium chloride concentration was reduced to 2.75 g/l.

The results summarized in Fig. 2 show that the dry weight per flask was slightly greater in the synthetic media. Therefore, the synthetic media is well suited for vegetative growth. In addition, in the synthetic media there was no significant difference in dry weight when the inoculum was increased by a factor of 10 or the calcium chloride concentration was reduced to 2.75 g/l. After 20 days, autolysis of the mycelial mats became evident and therefore, dry weight determinations were not extended past 24 days. From the results shown in Fig. 1 and 2 it may be noted that the complete synthetic medium with sucrose (Medium A, Fig. 1) supports good vegetative growth and asci production.

Of the different initial pH values employed in complete synthetic medium (Medium A, Fig. 1), only an initial

pH of 4.0 gave consistent results. Although the data is not reported in this dissertation, when an initial pH of 2.0 and 5.0 was used there was erratic asci formation. The higher ascospore counts at pH 2 as compared to pH 3 and 4 reported by Splittstoesser et al. (1969) may be due to the inherent differences between synthetic and non-synthetic media. From an initial pH of 4.0, a difference in pH values occurred during growth and asci formation between PDB and PSB media. The pH with PSB stabilized after 20 days at a pH of about 5.0 while the PDB showed a steady increase in pH values between 6.0 and 7.0. More rapid utilization of the glucose does not appear important since there is no appreciable difference in the rate of dry weight increase between PDB and PSB (Fig. 2).

The data in Fig. 3 and 4 show that peak asci formation, dry weight maxima, and stabilization of pH occurred approximately 4 days later in synthetic medium containing sucrose as compared to PSB although the same relative patterns can be observed. This suggests that some factor of factors may be present in the potato extract medium which may be responsible for triggering the earlier asci production. This factor (s) may not be syntesized in the synthetic medium until a later time. This situation may be similar to the dispensible but promoting growth factor of fungi described by Fries (1965). The synthetic medium appears suitable for studying asci formation by B. fulva

in terms of such growth or sporulation factors if they are involved, because such a factor(s) may alter the asci production curve in terms of total numbers and time. The effect of the $CaCl_2$ and the vitamins on sporulation alone cannot be determined because the effects on vegetative growth and sporulation could not be separated experimentally. Effects of the additives seems to be enhancement of growth through stabilization in addition to asci formation.

The percent of the asci population susceptible to heat shock appeared constant in the complete synthetic medium with sucrose from spore crop to spore crop and did not seem to vary over an extended period of time (Table 7). It appears that at any given time the heat shockable population does not increase appreciably in comparison to the total population. The low percent activation we have noted for B. fulva has been previously reported by Splittstpesser et al. (1969).

The purpose of this phase of our research was to determine the cultural requirements of ascosporogenesis in B. fulva in order to deliniate further the mechanism of ascospore heat resistance.

The procedure used to obtain purified asci suspensions allowed the preparation of asci with no more than 3% contamination by hyphae and conidia based on ascus to hyphal strand and ascospore to conidium ratios. The method utilized low speed centrifugation, differences in mass between

asci and contaminating material, and the innate ability of the asci to clump. These processes were combined to hold the heavier asci at a fairly constant level numerically while the lighter conidial and hyphal fragments were removed. A comparison of a partially purified asci suspension and a purified asci suspension may be made with Fig. 5 and 6. Photographs of a non-purified asci suspension were of a poor quality and therefore not included.

In terms of heat resistance B. fulva ascospores seem to be located between the heat resistant bacterial endospores and the generally heat sensitive fungal spores. The aspect of heat resistance is one of the most heavily worked and least understood disciplines in biology. To date five general theories are popular. These are: (1) the dynamic hypothesis (Allen, 1950), (2) the stable component hypothesis (Melitizer, 1949) (3) hydrogen bonding (Koffler, 1957), (4) converting principle or temperature factor (Sie, 1961), and (5) the inhibitor hypothesis (Swartz, 1957).

The possibility of lipids playing a role in the thermal resistance of fungi has recently been examined, especially in relation to the saturated fatty acids, sterols, and sterol esters (Mumma et al., 1970; Mumma et al., 1971; Mumma et al., 1971a). However, the content of saturated fatty acids as being due to the effect of growth temperature on lipids in general should be taken into consid-

eration (Sumner et al., 1969).

The implication of lipids in thermal stability is related to the stable component hypothesis (Melitzer et al., 1949) and is believed to affect the stability of membranes (Sussman and Halvorson, 1966; Mumma et al., 1970). The difference in the kinds of fatty acids in mesophilic and thermophilic bacteria has also been examined (Daron, 1970; Shen et al., 1970; Weerkamp and Heinen, 1972). The role of lipids in thermophily is based on the content and chain length of saturated fatty acids, since the elevation of lipid melting points by saturated fatty acids is well established (Leninger, 1971). The chain length in the cases of bacteria and fungi studies does not exceed 20 carbons to any significant degree. The importance of the longer chained fatty acids has been suggested in thermophiles (Shen et al., 1970) but has not previously been reported. Long chain fatty acids up through C27 have been reported for the sporophores of Fomes igniarius (Epstein, 1966) but their importance in thermal resistance has not been determined.

The results of the fatty acid analysis of asci, conidia, and hyphae of B. fulva are shown in Tables 8 and 8a. It may be noted that the saturated to unsaturated ratios for the C11 through C22 fatty acids for the asci, conidia, and hyphae are not significantly different. Actually the heat sensitive conidia and hyphae show a greater degree of

saturation in the C11 through C22 range than do the heat stable asci. The amount of branched chain saturated fatty acids in this range of chain lengths for any of the structures is insignificant.

A significant difference does appear in the fatty acids br-C26, C27, unknown #11 and unknown #12 extracted at 25 C (Table 1). These constitute 48.57% of the total fatty acids of the asci and 0.24% and 2.46% of the total fatty acids of the conidia and hyphae respectively. The significance of these results with respect to their contribution to the heat resistance of the asci (ascospores) is not clear at the present time. However, they may contribute to the heat resistance and dormancy of the B. fulva asci (ascospores) by providing a protective effect on cellular organelles.

Some observations not recorded in the results may be pertinent at this time. With light microscopy the ascus membrane enclosing the ascospores is very difficult to detect without the aid of dyes such as phyloxine, gram's iodine, and crystal violet. Dyes reveal no stainable material between the ascus membrane and the ascospores. After four days extraction at 25 C in chloroform:methanol viable ascospores can still be recovered, while no viable conidia can be recovered. Viable ascospores can still be recovered after two days extraction at 56 C. The asci are disrupted after 4 days extraction at 25 C resulting in the re-

lease of individual ascospores although the presence or absence of the ascus membrane could not be determined. Also very few non-refractile ascospores could be noted after the 25 C extraction although some internal structure could be noted in many and some ascospores lost their ellipsoidal shape.

Therefore the 56 C extraction (Table 8a) which followed the extraction at 25 C (Table 8) was essentially an extraction of individual ascospores. Upon extraction at 56 C changes in the fatty acid profile of the ascospores, when compared to the 25 C extraction were noted. Some fatty acids showed an increase in relative percent concentration as compared to the 25 C extraction, some showed a decrease and some did not change. The fatty acids which exhibited a significant increase were C14, C14:1, C16, C16:1, C18, unknown #9 and unknown #10; those showing a decrease were C18:1, br-C26, C27, unknown #11, and unknown #12; those showing no appreciable change were C18:2 and the remaining fatty acids which showed only trace amounts after either extraction of intact asci or ascospores. These results could indicate a difference in location and/or chemical bonding within the ascospore.

The next phase of the analysis was concerned with an analysis of the ascospores. This consisted of determinations of the spore coat dry weight/spore, ascospore coat lipid content, and total ascospore lipid composition.

The ascospore coat was shown to make up 60% of the dry weight of the ascospore (Table 9) which is in the range of the bacterial endospore coats (Tipper and Gauthier, 1972). The ascospore lipid content was found to be 10.50% (Table 9) which is higher than lipid values reported for bacterial endospore coats (Sussman and Halvorson, 1966a). The results of the total lipid analysis of the ascospores showed 22.75% (Table 9) of the ascospore consists of lipid which is in the upper range for bacterial endospores (Murrell, 1969) and the bulk of the lipid is located in the sporoplasm. Analysis of the lipid classes revealed no sterol or sterol esters although they have been reported in some thermophilic fungi (Mumma et al., 1971). The role of the two unknown lipid classes found in the B. fulva ascospores is unknown.

Based on observations of the thinness of the ascus membrane, the lack of stainable material between the ascus membrane and the ascospores, the low lipid content of bacterial spore exosporia (Tipper and Gauthier, 1971) and the observation of fatty acid variation between the extractions at 25 C (Table 8) and 56 C (Table 8a), it appears that most if not all of the lipid is located in the ascospore. Based on this observation the next phase of the research involved the rupturing of intact asci to obtain a non-soluble portion (designated ascospore coats) and a soluble supernatant fraction (designated sporoplasm) in an

attempt to determine if any of the lipid classes (Table 10) and fatty acids (Table 11 and 12) were located mainly in the ascospore coat or sporoplasm. The lipid analysis was qualitative and the total lipid and fatty acid analysis was quantitative. The results indicated preferential location of fatty acids within either the coats or the sporoplasm, suggesting a difference in function for the fatty acids. The results revealed that the greatest amount of the saturated fatty acids were present in the sporoplasm and the fatty acids with the greatest chain lengths (br-C26, C27, unknown #11 and unknown #12) were located in the coat, suggests a very hydrophobic nature for these two major portions of the ascospore. The presence of lipids in bacterial endospore coats has been suggested as the basis for the hydrophobic nature of some bacterial spores (Sussman and Halvorson, 1966a). By similar reasoning, the asci and ascospores of B. fulva would seem to be very hydrophobic. This is also indicated by the need for Tween 80 in the purification procedure to keep the asci and ascospores in suspension.

None of the saturated fatty acids follow the distribution of the C18:1 and C18:2 fatty acids which showed almost an equal distribution between the ascospore coats and sporoplasm fraction. Since the C18:1 and C18:2 fatty acids are commonly associated with membranes, these results suggest that the saturated fatty acids may not be inte-

grally associated with the cytoplasmic membrane. This would not be in agreement with the lipid theory of thermophily which places saturated fatty acids as integral components of the membranes in order to confer stability at elevated temperatures. If any lipids are involved in the heat resistance of the ascospores, the fatty acids would not seem to be an integral part of the heat resistance system at the cytoplasmic membrane level.

The importance of the fluorescent band and the hydrocarbon in the ascospore sporoplasm can not be explained at this time.

The final phase of the lipid analysis was the determination of the fatty acid profiles of the individual lipid classes except for the hydrocarbon class. These results are given in Tables 13 through 19. As mentioned in the results, each class has at least one salient feature which distinguished it from every other class. These results may mean differences in function for the lipid classes. Only the diglyceride class showed a difference in the sporoplasm and the ascospore coat fractions suggesting a different function for each of these fractions. Due to the high concentration of lipid in the ascospores and the diversity of the fatty acid profiles of the individual classes, the function of the lipids appears to be extremely complex.

As shown in Fig. 9, 10, and 11 some of the B. fulva

ascospore resemble bacterial spores (Sussman and Halvorson, 1966; Fitz-James and Young, 1968) more than they do typical ascospores (Lowry and Sussman, 1967; Bandoni et al., 1966; Moens, 1971) in terms of the thick spore coat (outer coat and inner coat) and the condensed cytoplasmic material. Many ascospore sections resembled Fig. 9 except the nuclear membrane was not detectable. In comparison to the B. fulva conidium (Fig. 7) the ascospore coat is much thicker.

The unit membrane structure surrounding the sporoplasm is clearly observable in Fig. 10,11 and 12. However, it should be noted in these figures that the membrane is irregularly folded, which is atypical of ascospore. Such a membrane may not have full biological activity. Fig. 9 and 10 show that the irregular membrane structure is not merely due to dehydration since most of the cytoplasmic membrane is in contact with the inner spore coat. It should be considered that this folding of the membrane, regularly present in the ascospore thin section, may be an artifact of the dehydration procedure. If there was dehydration, the section in Fig. 8 does not show the effect. Fig. 8 is more representative of ascospores in general appearance, but still indicates an unusually thick ascospore coat.

Of interest are the inclusion bodies evident in Fig. 9, 10, 11, and 12. It may be noted that these are always

found in fairly close association with the cytoplasmic membrane. As shown in Fig. 8, there is structural differentiation and these inclusion bodies are not present. Also the cytoplasmic membrane does not have a "stacked" appearance. It is possible that these inclusion bodies may be some form of structural differentiation inhibitor and/or an energy source. Since saturated fatty acids and hydrocarbons tend to predominate in the sporoplasm rather than the ascospore coat, it is possible that these light staining areas may be saturated fatty acids or hydrocarbon. If these inclusion bodies are either or both of these, in such association to the cytoplasmic membrane, this hydrophobic area may cause or aid in the "stacking" or infolding of the cytoplasmic membrane. The infolding may be due to the attraction of the hydrophobic groups of the membrane and the inclusion bodies. It may also be noted that these inclusion bodies stain similarly to the inner coat material, so they may merely be projections of the inner coat into the cytoplasm.

Fig. 7 is a thin section through a conidium. The conidium shows no unusual features as do the ascospores in terms of coat thickness and condensed cytoplasm. One may note a very complex cytoplasmic membrane with much evidence of invagination. Particularly interesting is the structure labeled pm. This appears to be an infolding into the cytoplasm of the inner membrane of the cytoplasmic unit mem-

brane. It was designated plasmalemmasome-like and this type of structure has not been reported for conidia in the ascomycetes.

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Vita

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EXAMINATION AND THESIS REPORT

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Major Field: Microbiology

Title of Thesis: Byssochlamys fulva; nutrition of ascospore formation; fatty acid profiles of ascospores, conidia, and mycelia; and electron microscopy of ascospores and conidia.

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